Processing Lignocellulosic Biomass into Ethanol - Implications of High Solid Loadings

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Implications of High Solid Loadings

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“To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge”

Nicolaus Copernicus

Abstract

Fuel ethanol from lignocellulosic biomass has the potential to provide a sustainable replacement for traditional oil-based fuels. This dissertation assesses the processing of three different lignocellulosic materials – spruce, wheat straw and giant reed – at industrially relevant solid loadings. The work is divided into two main parts. The first part deals with the degradation of biomass to sugars, focusing on the complex rheological behavior of biomass slurries and the connection to mixing during high solids hydrolysis. The second part deals with the process design of combined hydrolysis and fermentation processes, focusing on efficient xylose co-consumption at high solids loadings.

Rheological characterization of steam pretreated spruce revealed strong non-Newtonian flow behavior with rapidly increasing viscosities and yield stress at high solid loadings, for instance the yield stress more than doubled when increasing the WIS content from 10 to 12 % (from 10 Pa to 24.5 Pa). Moreover, a strong effect of particle size distribution was found on both the viscosity and the yield stress. High viscosities create a need for efficient mixing during enzymatic hydrolysis of pretreated spruce. The hydrolysis rate was significantly influenced by both the amount and type of agitation. For pretreated spruce, for example, an increased agitation rate from 75 rpm to 500 rpm doubled the hydrolysis yield after 96 hours (an increase in yield from 35 to 72 %). The positive effect remained during scale-up to cubic meter scale and could be correlated to the flow conditions in the reactor. However, large discrepancies were found between different pretreated materials, and it became evident that the hydrolysis rate of giant reed was not affected by mixing. This was likely due to the much more rapid liquefaction achieved during the hydrolysis of giant reed.

In addition to glucose, many potential raw materials contain considerable amounts of the pentose sugar xylose. Xylose metabolism has today been successfully implemented in *Saccharomyces cerevisiae* through genetic engineering, although glucose is still the preferred substrate. In this work, xylose co-consumption was significantly enhanced by applying different process design strategies. By using a dual feed strategy, xylose consumption could be increased by 25 %, which resulted in a 10 % increase in final ethanol titer. It was also found that, in the presence of high acetic acid concentrations, xylose uptake could be significantly enhanced by increasing the pH. Whether or not this was beneficial for ethanol production, however, was found to be dependent on the specific process design.

Populärvetenskaplig sammanfattning

Ansträngningen att framställa nya förnyelsebara drivmedel har under de senaste årtionden ökat markant. Anledningarna har varit flera, dels oron för klimatpåverkan vid användningen av fossila bränslen, dels från ett nationellt energisäkerhetsperspektiv då lokal produktion skulle kunna stabilisera tillgången. Bioetanol är ett av de största biobränslena i världen och produceras idag framförallt från sockerrör och majsstärkelse. När man betraktar totala växthusgasutsläpp är i synnerhet majsstärkelse i längden nödvändigtvis inte hållbart. Analyser visar att ett mer hållbart alternativ är att använda hela växtmaterial och inte bara frö-delen som det görs idag. Omvandlingen av växtmaterial är dock en mycket mer komplicerad process där materialet först måste brytas ned enzymatiskt till fria sockermolekyler som sedan jäses till etanol. Vidare består växtmaterial generellt av flera olika sockerarter. Den mest förekommande sockerarten är glukos, som kan jäsas till etanol naturlig av vanlig bagerijäst, *Saccharomyces cerevisiae*, vilken traditionellt används inom etanolindustrin. För att jäsa xylos, den näst mest förekommande sockerarten i många växtyper, krävs däremot genmodifiering av jästen.

Forskningen har nu kommit så långt att de första kommersiella anläggningarna har börjat tas i drift. För dessa anläggningar är vikten av att kunna arbeta vid höga torrhalter stor, då detta förutspås ge stora ekonomiska fördelar i form av lägre investerings- och driftskostnader. Att arbeta vid höga torrhalter är dock en stor processteknisk utmaning, bland annat på grund av hög viskositet samt höga koncentrationer av fermentationsinhibitorer.

Arbetet i denna avhandling har syftat till att förstå den komplexa reologin, dvs. flödesegenskaperna, hos förbehandlat granmaterial. Dessutom kopplas dessa till hur omblandning påverkar den enzymatiska nedbrytningen av materialet till socker. Flödesegenskaperna visade sig vara starkt kopplade till mängden torrmaterial samt till fördelningen av fiberstorlek i materialet. Vidare påvisades en stark inverkan av omrörning på den enzymatiska hydrolysen av förbehandlad gran, vilket kunde kopplas till flödesbetingelserna i reaktorn. För mer gräslika material kunde dock ingen påverkan av omrörning påvisas. Detta var säkerligen kopplat till den mycket snabbare förvätskning som skedde under den enzymatiska hydrolysen.

Nya processtrategier utvecklades dessutom för att med hjälp av genmodifierade jäststammar effektivare omvandla både glukos och xylos till etanol. Genom att mata reaktorn med både material och enzymer så kunde xylosupptaget i processen ökas med 25 %, vilket gav en 10 %-tig ökning av den slutliga etanolkoncentrationen. Vid höga koncentrationer av ättiksyra kunde dessutom xylosupptaget ökas markant genom en ökning av pH.

List of publications

This dissertation is based on the following publications, which will be referred to in the text by their Roman numeral:

I. Wiman M, **Palmqvist B**, Tornberg E, Liden G. (2011) Rheological characterization of dilute acid pretreated softwood. Biotechnol Bioeng. 108:1031 – 41

II. **Palmqvist B**, Wiman M, Liden G. (2011) Effect of mixing on enzymatic hydrolysis of steam-pretreated spruce: a quantitative analysis of conversion and power consumption. Biotechnol Biofuels 4:10.

III. **Palmqvist B**, Lidén G. (2012) Torque measurements reveal large process differences between materials during high solid enzymatic hydrolysis of pretreated lignocellulose. Biotechnol Biofuels 5:57

IV. Kadić A, **Palmqvist B**, Lidén G. Effects of agitation on particle-size distribution and enzymatic hydrolysis of pretreated spruce and giant reed (S*ubmitted*)

V. **Palmqvist B**, Kadić A, Hägglund K, Petersson A, Lidén G. Mixing considerations when scaling up enzymatic hydrolysis of pretreated spruce. (*Submitted*).

VI. Olofsson K, **Palmqvist B**, Liden G. (2010) Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. Biotechnol Biofuels 3:17

VII. **Palmqvist B**, Lidén G. Combining the effects of process design and pH for improved xylose conversion in high solids ethanol production from Arundo donax. (*AMB Express – Accepted*)

I have also contributed to the following book chapter (not part of this dissertation):

Mutturi S, **Palmqvist B**, Lidén G. Developments in bioethanol fuel-focused biorefineries, In Advances in biorefineries, Ed. K. Waldron, Woodhead Publishing, Cambridge, *in print*, (2014). (ISBN 9780857095213)

My contributions to the publications

I. I participated in the design of the study, the experimental work and the discussion about the results. I was involved in the preparation of the manuscript.

II. I participated in the design of the study, performed the experimental work and wrote the manuscript.

III. I designed the study, performed the experimental work and wrote the manuscript.

IV. I participated in the design of the study, the experimental work and the discussion about the results. I was involved in the preparation of the manuscript.

V. I participated in the design of the study, performed the lab-scale part of the experimental work and wrote the manuscript.

VI. I participated in the design of the study, the experimental work and the discussion about the results. I was involved in the preparation of the manuscript.

VII. I designed the study, performed the experimental work and wrote the manuscript.

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Sist men inte minst, tack till min underbara familj:

Mamma, Pappa och lillsyster, tack för att ni alltid finns nära till hands och stöttar!

Alison, my angel! Thank you for always being there for me. Je t'aime!

Abbreviations

ADP – Adenosine Diphosphate

AFEX – Ammonia Fiber Explosion

ATP – Adenosine Triphosphate

BG – β-Glucosidases

CBH – Cellobiohydrolases

CBM – Cellulose Binding Module

CDH – Cellobiose de-Hydrogenase

DM – Dry-Matter

DOE – (US) Department of Energy

DP – Degree of Polymerization

EG – Endo-1,4-β-Glucanases

EISA – Energy Security and Independence Act

FAD/FADH2 – Flavin Adenine Dinucleotide

FP7 – 7th Framework Programme

FPU – Filter Paper Unit

GHG – Green House Gas

GRAS – Generally Regarded As Safe

HMF – 5-Hydroxymethyl-2-Furaldehyde

HPLC – High-Performance Liquid Chromatography

IEA – International Environmental Agency

LPMO – Lytic Polysaccharide Monooxygenase

NADH/NAD+ – Nicotinamide Adenine Dinucleotide

NADPH/NADP+ – Nicotinamide Adenine Dinucleotide Phosphate

NREL – National Renewable Energy Laboratory

PPP – Pentose Phosphate Patway

PSD – Particle Size Distribution

RED – Renewable Energy Directive

RPM – Revolutions Per Minute

SEM – Scanning Electron Microscopy

SHCF – Separate Hydrolysis and co-Fermentation

SHF – Separate Hydrolysis and Fermentation

SSCF – Simultaneous Saccharification and co-Fermentation

SSF – Simultaneous Saccharification and Fermentation

STEX – Steam Explosion

TCA – Tricarboxylic Acid

WIS – Water Insoluble Solids

XDH – Xylitol Dehydrogenase

XI – Xylose Isomerase

XK – Xylulokinase

XR – Xylose Reductase

Contents

Chapter 1

Introduction

In recent years, environmental concerns related to increased average temperatures and rising atmospheric carbon dioxide levels have prompted much debate about our use of fossil fuels. About 25 % of global carbon dioxide emissions comes from the transportation sector, with three quarters of that being attributed to road transport (IEA 2009). Additionally, there is a striking difference in the use of cars around the world. In the US, for example, there were about 0.7 cars per capita in 2006, compared to only 0.02 cars per capita in China (Goldemberg 2006). With the great economical expansion in China this difference will very likely decrease rapidly. This factor, coupled with the expected increase in global population to an estimated 8.3 billion by 2030 (Arundel and Sawaya 2009), means energy needs for transportation could double by 2050 (IEA 2009). The need for a more sustainable and renewable fuel is thus evident and imperative.

There is a variety of potential biofuels that could be produced and used to replace petroleum based fuels, including bioethanol, biogas, biodiesel and biobutanol. Each fuel clearly has its own virtues and disadvantages and a combination of them will likely be needed in order to efficiently replace oil. The focus of this dissertation is on the efficient production of bioethanol, which today is one of the largest biofuels on the market, having replaced about 3 % of the fossil-based gasoline consumed in the world (Goldemberg 2008). Of course, biofuels are only one, admittedly important, part of the solution to a more sustainable future. Other important factors include the use of more energy efficient vehicles and changes to our transportation systems in general.

1.1 Fuel ethanol – History and outlook

Almost all fuel ethanol today is produced from sugar (sugar cane) or starch (mainly corn), through so called '1st generation' bioethanol processes. Two countries, namely Brazil and the US, dominate the market (Figure 1.1). Historically, Brazil was the main producer of fuel ethanol, starting with the launch of the Pro-Alcohol program in the mid 1970s (Goldemberg 2006). However, in 2005, the US surpassed Brazil as the world's leading producer of bioethanol and since then the production gap has only widened (Figure 1.1).

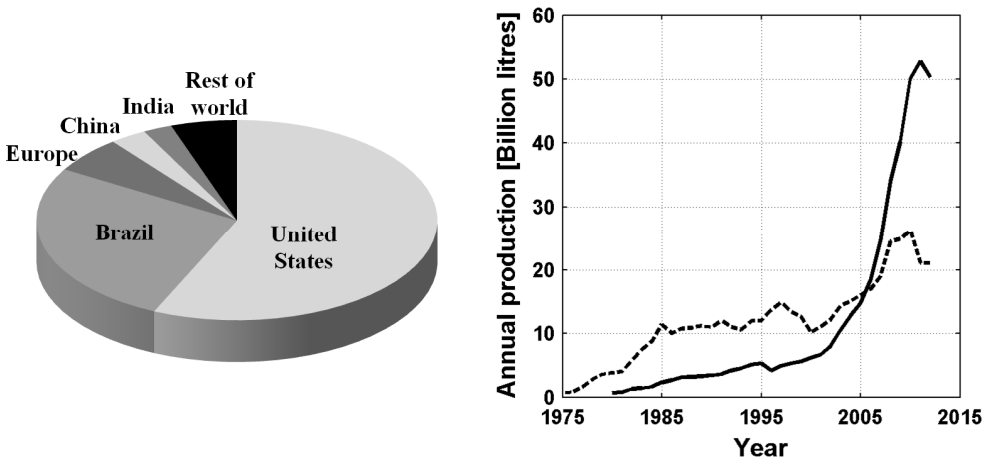

Figure 1.1. Total world production of bioethanol in 2012 divided between the major contributors (Left) and annual fuel ethanol productions by Brazil (dotted line) and the US (solid line) (Right). Source: The Renewable Fuels Association[1] and Goldemberg 2006.

The production of conventional (1st generation) bioethanol has been increasingly questioned for competing for agricultural land with food and feed production. Therefore, the issue is no longer only how to replace fossil fuels, but how to do this in a sustainable manner (Börjesson 2009). This has shifted focus away from using conventional agricultural raw materials to using lignocellulosic biomass, also known as '2nd generation' or advanced biofuels. In both Europe and the US, there are strong political incentives to introduce advanced biofuels on the market. The European Union, for example, has put forth the 20-20-20 targets aiming at increasing the share of renewable energy to 20 % (10 % in transportation fuels), improving energy efficiency by 20 % and reducing green house gas (GHG) emissions by 20 %, all by the year 2020. Sustainability criteria for biofuels

[1] http://www.ethanolrfa.org (2014-03-19)

counting towards these goals are set in the Renewable Energy Directive, RED (Directive 2009). The RED mainly addresses GHG savings and the protection of land with high biodiversity, while also providing incentives to stimulate advanced biofuels production from waste, residues and lignocellulosic material (Janssen et al. 2013).

In the US, more direct volume targets have been put forward in the Energy Security and Independence Act of 2007 (EISA). Mandates are in place to reach specific production levels over time until reaching 136 billion liters in 2022, of which 79 billion liters must be produced from feedstocks other than grain (Janssen et al. 2013).

Both the US and EU provides financial support to advanced biofuels projects. In February 2012, the US Department of Energy (DOE) granted a total funding of US$766 million to 16 cellulosic ethanol projects, ranging from pilot to commercial scale (Balan et al. 2013). In the EU, financial support has been given to industrial demonstration projects through the 7th Framework Programme (FP7) and more recently, some €82 million were allocated through the NER300 program to demonstration projects utilizing the biochemical route (Balan et al. 2013).

Even though commercialization has now started, the technology to refine lignocellulosic biomass to ethanol is still regarded as costly. Further process development, especially concerning concentrated biomass utilization, is therefore crucial in order to make lignocellulosic fuel ethanol cost competitive. With these efforts, a learning curve similar to for example the Brazilian ethanol industry, where production cost have dropped 3-4 fold since the start (Goldemberg 2008), can be expected.

1.2 Scope and outline of the dissertation

The work carried out within this dissertation can be divided into two main parts, each highlighting a specific part in the production chain of refining lignocellulosic biomass. The first part deals with the production of sugar from highly concentrated biomass slurries, focusing on understanding the implications of high solids handling during enzymatic hydrolysis. The hydrolysis of biomass into sugars is in fact generic to any kind of sugar based biorefinery, and not specific to ethanol production. The second part deals with the fermentation of the produced sugars to ethanol. Here, the focus is on designing process strategies for improved xylose conversion.

The main objectives of the work were to:

- characterize the rheology of pretreated spruce and investigate its implications on the enzymatic hydrolysis
- understand how mixing affects enzymatic hydrolysis of different types of pretreated biomass
- enhance xylose conversion during co-fermentation of glucose and xylose through process design

This dissertation is structured according to the two main subjects studied. First, in Chapter 2, the lignocellulosic biorefinery process is outlined together with a description of the raw material and a brief overview of a generic ethanol-based biorefinery. Chapter 3 describes the production of sugars from high solids biomass and presents findings on rheological changes, agitation effects and scale-up issues during enzymatic hydrolysis of lignocellulosic biomass. In Chapter 4, the fermentation process is discussed, with focus on process designs for enhanced xylose conversion. The final chapter, Chapter 5, summarizes the main findings and outlines suggestions for further work within this field.

Chapter 2

The lignocellulosic biorefinery

A biorefinery can be seen as an analog to the more common oil-based refineries, where crude oil is fractionated and converted into a multitude of products – many of them fuels. The main difference is of course the raw material, which in a biorefinery is biomass, preferably lignocellulosic biomass. The variety of products coming out of a biorefinery is also different compared to an oil-based refinery. The intention, however, is to produce fuels and/or platform chemicals which would fit nicely into the current chemical industry. Some of the potential platform chemical candidates for this purpose were listed by the US Department of Energy (DOE) in the "top 10 list", which was revised in 2010 by Bozell and Petersen (2010). Lignocellulose can be refined either thermo-chemically, through for example gasification or pyrolysis, or biochemically through enzymatic hydrolysis and fermentation (the so called 'sugar-platform'). This dissertation focuses on the latter, bioethanol production through the sugar platform. Production of ethanol from lignocellulosic biomass can certainly be seen as the first step towards full-fledged biorefineries. This is especially due to ethanol now often being considered as one of a range of products in the conversion of biomass, which is the very basic idea of a biorefinery (Pham and El-Halwagi 2012).

This chapter is intended to give the reader a brief overview of the structure of the lignocellulosic material and introduce the reader to the core process steps in a sugar platform biorefinery where ethanol is one of the main products.

2.1 The raw material

The lignocellulosic material differs greatly in structure compared to for example starch, which is currently used as a source of sugar in the US ethanol industry. In Europe, wheat starch is one of the main sources of sugar for fuel ethanol production, as in the Agroetanol plant[2] in Norrköping, Sweden. The difference between starch and cellulose – or lignocellulose – is related to the significantly different function of the two materials in nature. Starch serves as energy storage for plants and hence needs to be rather easily degradable, whereas lignocellulose comprises the construction material for the structure of plants. Lignocellulose thus has to be resistant to both mechanical wear and microbial and chemical degradation.

The main part of the cell wall in all plant material is built up by lignocellulose. In turn, the lignocellulosic biomass is mainly composed of three types of macromolecules, namely cellulose, hemicelluloses and lignin. Cellulose is the most abundant polymer and constitutes the largest reservoir of organic carbon on earth, with an estimated annual production in plants of about 180 billion tons (Festucci-Buselli et al. 2007). This makes many kinds of lignocellulosic materials interesting feedstock options for biorefinery purposes. Both agricultural and forestry residues, as well as different grasses and energy crops, have been studied for the purpose of biorefining. However, the feedstock(s) to choose will depend mostly on regional availability, but also on market prices and political decisions. The choice of feedstock will to a certain extent dictate the whole refining process since the ratio and structure of the different polymers varies within relatively large limits between different biomass types (Table 2.1).

[2] http://www.agroetanol.se/en/ (2014-03-27)

Table 2.1. Typical composition of different lignocellulosic biomass. (* NR – not reported)

	Glucan	Xylan	Mannan	Arabinan	Galactan	Lignin
Crop residues						
Wheat straw[1]	32.6	20.1	0	3.3	0.8	24.2
Corn stover[2]	36.0	19.8	NR*	2.8	1.3	17.8
Sugarcane Bagasse[3]	43	26	NR	1.5	0.4	22
Softwoods						
Spruce[4]	49.9	5.3	12.3	1.7	2.3	28.3
Pine[5]	43.6	6.6	10.8	1.6	2.2	26.8
Hardwoods						
Willow[6]	43	14.9	3.2	1.2	2.0	26.4
Poplar[2]	39.8	14.8	2.4	1.2	NR	26.9
Energy crops/grasses						
Switchgrass[2]	32.2	20.3	0.4	3.7	NR	19.5
Giant reed[7]	35.7	18.6	0.2	1.6	0.6	22.3

[1] (Linde et al. 2008), [2] (Esteghlalian et al. 1997), [3] (Rudolf et al. 2008), [4] (Söderström et al. 2003), [5] (Frederick Jr et al. 2008), [6] (Sassner et al. 2006), [7] (Scordia et al. 2011)

2.1.1 Biomass structure and composition

The plant cell wall is built up by several layers, each with a different distribution of cellulose, hemicelluloses and lignin. The middle lamella (ML) is the outer layer of the cell wall. This thin layer is highly lignified and serves to connect or attach the different cells to each other. Inside of the ML is the primary cell wall (P), a thin layer of highly lignified cellulose fibrils oriented in all directions (O'Sullivan 1997) and embedded in a matrix of hemicelluloses and pectin. The primary cell wall is similar in different cell types (Brett and Waldron 1996). The secondary cell wall, however, differs considerably between different cell types (Brett and Waldron 1996) and is segmented into three different layers, a thin outer and inner layer (S1 and S3) and a thick middle layer (S2). The secondary cell wall is less lignified. The thicker S2 layer makes up the major part of the cell wall and comprises the major part of the carbohydrates (Figure 2.1). The main structural difference between the S layers is the microfibril orientation. Inside of the secondary cell wall is the warty layer (WL), a thin membrane containing warty deposits (Sjöström 1993).

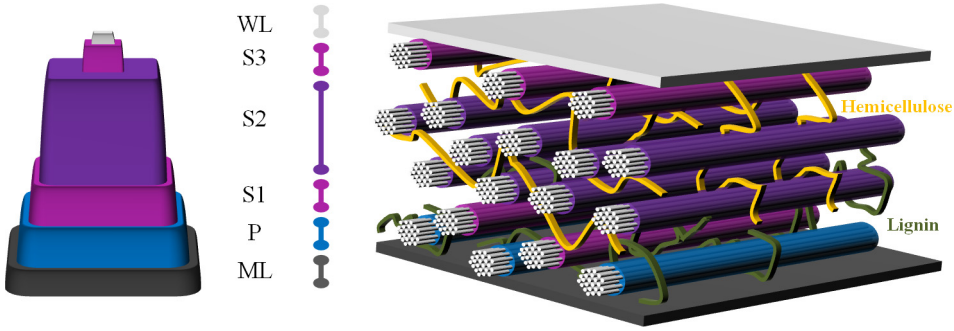

Figure 2.1. Structure of a typical plant cell (left) and the lignocellulose structure within it (right).

Cellulose is made of repeating units of cellobiose, linked by β-1,4-glycosidic bonds. Cellobiose, in turn, consists of two glucose molecules connected by a β-1,4-glycosidic bond. The degree of polymerization (DP) ranges between 300 to 15 000 for different species, but is usually around 8 000 for softwood cellulose (Fengel and Wegener 1989) and typically a bit higher in straw materials (O'Sullivan 1997). These linear polymer chains form sheets due to strong hydrogen bonding. The sheets are held together by hydrogen bonding and hydrofobic interactions (Lindman et al. 2010; Yamane et al. 2006). These sheets aggregate to form microfibrils, about 30-100 polymer chains, containing both crystalline and amorphous regions (Beguin and Aubert 1994; Sjöström 1993). The microfibrils are then packed up to form fibrils which finally forms the cellulose fiber.

Contrary to cellulose, the hemicellulose macrostructure is branched and has a much lower degree of polymerization, typically around 80-200 (Sjöström 1993). The hemicelluloses are bound to cellulose by hydrogen bonds and tend to form a matrix network entangling the cellulose fibers, providing the structural backbone of the cell wall (Mosier et al. 2005). The hemicelluloses are built up by a number of different sugar monomers such as D-xylose, D-mannose, D-glucose and L-arabinose. The main part of softwood hemicelluloses is typically built up by linear, or slightly branched, galactoglucomannans (Fengel and Wegener 1989; Sjöström 1993), whereas the dominant component in straw and grass hemicelluloses are arabino-glucuronoxylan, glucorono-arabinoxylan and arabinoxylan (Gírio et al. 2010) (Table 2.1). The hemicellulose components are furthermore O-acetylated.

Lignin, the second most abundant polymer in plants, fills the space in the cell wall by cross-linking different polysaccharides. It is covalently bond to hemicelluloses and also cellulose by ether, ester and glycosidic bonds (Ralph et al. 2004), providing structural support to the plant. Moreover, lignin plays a crucial part for water conduction in plants due to its hydrophoicity, which provides an obstacle for water adsorption to the cell wall. Lignin is a polyaromatic, highly branched, molecule consisting of three major phenylpropane compounds, i.e. p-

hydroxyphenyl (H), guaiacyl propanol (G) and syringyl propanol (S). The compounds are in turn synthesized from p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol respectively (Pérez et al. 2002; Ralph et al. 2004; Sjöström 1993).The proportions of the building blocks vary significantly among different species, where G is the main unit in softwood and G and S the most common in hardwood, while all three are present in straw and grasses (Sun et al. 1997). Furthermore, variations in lignin content and composition vary within the different cell types in the plants.

Besides cellulose, hemicelluloses and lignin, the biomass also contains many other compounds, for example pectins, fats, resin acids, proteins and inorganic compounds, but in lower mass fractions than the three main macromolecules (Fengel and Wegener 1989; Sjöström 1993).

In this dissertation, three different materials have been used; spruce (*Picea abies*), wheat straw (*Triticum aestivum*) and giant reed (*Arundo donax L.*). Wheat straw and giant reed are rather similar in composition and structure since they are both grass materials. Spruce on the other hand differs quite a bit from the other two in terms of both composition and structure of the main polymers. The cellulose polymer, for example, generally has a shorter DP for softwoods whereas wheat straw, for instance, tends to contain less crystalline regions (Liu and Sun 2010). For the hemicelluloses, mannose is the main sugar in spruce, whereas xylose dominates in the other two materials. The hemicellulose polymer also tends to be less branched and less acetylated in softwoods (Fengel and Wegener 1989; Sjöström 1993). The lignin content is typically higher in softwoods compared to grass materials, but perhaps more importantly, the composition of lignin differs to a great extent between spruce and straw materials. Spruce mainly contains lignin type G, while grass materials typically contain a mix between G, S and H lignin. As will be discussed later in this dissertation, these differences in structure and composition affect the process configuration.

2.2 The ethanol producing biorefinery – A brief overview

After a brief discussion on the raw material, this dissertation now turns to the process of converting biomass to the desired products, in this case ethanol. The main process steps in an ethanol biorefinery will be the same regardless of the raw material used and what kind of by/co-products are produced in the process. The operation of each of the core steps will, however, differ depending on both feedstock and product distribution. The process steps are:

- pretreatment
- enzymatic hydrolysis
- fermentation

In addition to these three core processes, product recovery (mainly distillation) and wastewater treatment (usually including an anaerobic digestion step to produce biogas) are needed. Distillation is a mature technique, with long gained experience from, for example, 1st generation bioethanol production. It will therefore not be discussed further in this work. Wastewater treatment also falls outside the scope of this dissertation, although this is certainly important within biorefineries. Even though pretreatment, hydrolysis and fermentation are always needed, the way to operate each individual step is highly dependent upon the feedstock selection and the combination of by/co-products in the biorefinery.

An attractive type of biorefinery, especially in the Nordic countries, is the energy focused refinery where ethanol is accompanied by for example the production of electricity, biomass pellets, biogas and perhaps district heating, if the location permits. A process setup of a generic energy focused biorefinery is shown in Figure 2.2. For the remaining part of Chapter 2, the fundamentals of pretreatment, enzymatic hydrolysis and fermentation will be further discussed.

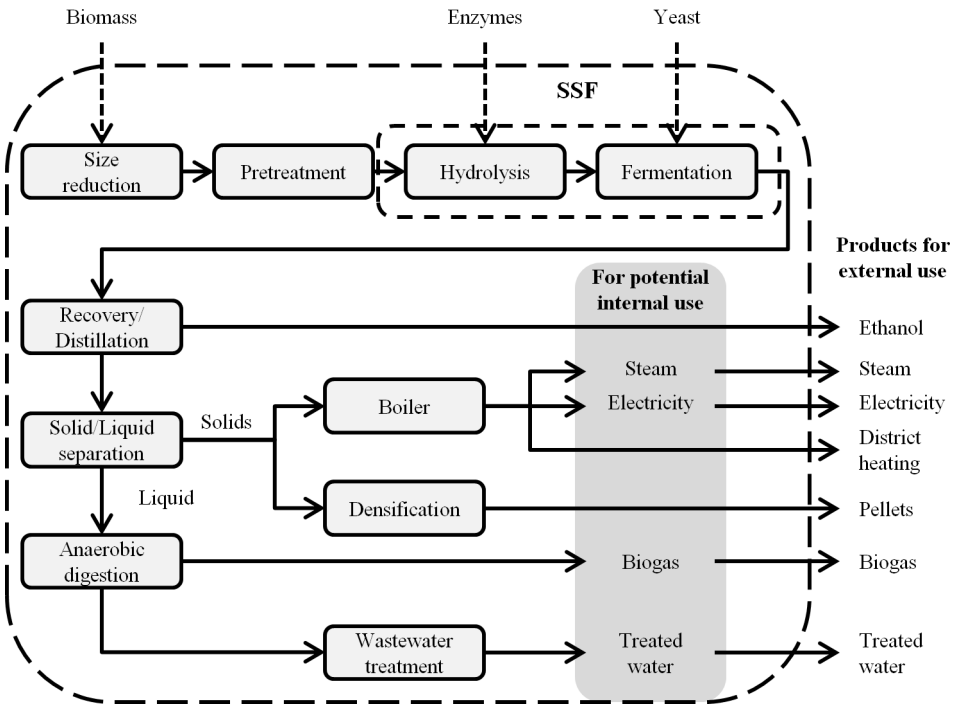

Figure 2.2. Overview of a generic energy focused biorefinery

2.2.1 Pretreatment

Due to the recalcitrant nature of lignocellulosic biomass, pretreatment is necessary in order to effectively hydrolyze the fibrous cellulose into monomeric sugars. Pretreatment is one of the most expensive process steps in the conversion of biomass to ethanol (Mosier et al. 2005; Sassner et al. 2008) and it affects all the subsequent steps in the production, from hydrolysis to fermentation and all the way down to wastewater treatment.

Pretreatment of the biomass aims to open up the structure of the fibers and/or to dissolve parts of the material, primarily either the hemicelluloses or the lignin. The reasons for the improvements in digestibility gained through pretreatment are not fully understood. However, changes are induced in for example crystallinity, degree of polymerization (DP) and accessible surface area of the fibers. All these factors are believed to affect the rate of enzymatic hydrolysis (Zhang and Lynd 2004).

Numerous pretreatment methods exist. They are commonly divided into physical and chemical methods depending on their main mode of action, although a combination of the two is often used (Alvira et al. 2010; Galbe and Zacchi 2007; Mosier et al. 2005). Physical methods, such as communition and extrusion, rely on

size reduction and defibrillation of the material. This is as a way to open up the fiber structure and create a larger accessible surface area to enhance the enzymatic hydrolysis. Purely physical methods are typically very energy intensive and are therefore often regarded unfeasible (Hendriks and Zeeman 2009). However, in combination with other pretreatment methods they can be useful (Galbe and Zacchi 2007).

Chemical pretreatments generally aim at removing, either the hemicelluloses or the lignin part of the biomass, by dissolution. Acid is commonly used to dissolve hemicelluloses, whereas lignin is typically dissolved by alkaline or organosolv pretreatments (Alvira et al. 2010; Galbe and Zacchi 2007). Recovery of the chemical catalyst is often crucial for these processes (Mosier et al. 2005).

Another pretreatment method which has received a lot of attention recently is the use of ionic liquids. Ionic liquids are salts that have low melting point and hence behave as liquids at low to moderate temperatures. They are typically composed of large organic cations and small inorganic anions. The benefits of ionic liquids are that they effectively dissolve both cellulose and lignin, with minimal sugar losses, while operating at relatively low temperatures. The main drawback is the cost of the ionic liquids, which requires very efficient recycling methods for ionic liquids to become industrially interesting (Alvira et al. 2010).

A promising physiochemical pretreatment method is ammonia fiber explosion (AFEX). Biomass is treated with liquid ammonia under pressure, at relatively low temperature. Releasing the pressure leads to a rapid expansion, this result in physical disruption and swelling of the biomass. During pretreatment, a partial deacetylation is achieved. AFEX, however, does not remove either lignin or hemicelluloses to any greater extent, resulting in a need for hemicellulases during hydrolysis. Reduction in unproductive binding of cellulases to lignin has been reported using AFEX pretreatment (Alvira et al. 2010). AFEX is considered promising in particular for agricultural residues and herbaceous crops, but not very efficient against softwoods, presumably due to their high lignin content (Alvira et al. 2010; Chandra et al. 2007; Galbe and Zacchi 2007; Mosier et al. 2005). One of the main advantages of AFEX is the high sugar recovery, while an efficient recovery of the ammonia remains a large challenge.

Steam explosion (STEX)

One of the most studied pretreatment methods is steam explosion (STEX). This is also the method used for the different materials within this work and will therefore be presented in more detail here.

STEX is a physiochemical pretreatment method, in which the material is exposed to high pressure saturated steam (typically 160-230 ˚C) for a few minutes, with or without the addition of an acid catalyst. The term ‘steam explosion’ originates from the rapid release of pressure, which was initially thought to open up the

structure of the material. It has, however, later been shown that the pressure release only has a minor effect on the enzymatic digestibility of the material (Galbe and Zacchi 2007; Kumar et al. 2009; Mosier et al. 2005). The main effect has instead been attributed to the removal of hemicelluloses by either the added acid or through auto-hydrolysis, caused by the released acetic acid from the material (Galbe and Zacchi 2007). Lignin is not removed to any large extent with STEX, but part of the lignin is redistributed on the fiber surface as a result of melting and repolymerization (Alvira et al. 2010; Kumar et al. 2009), see Figure 2.3. Steam explosion has proven useful on a wide variety of substrates including agricultural residues (e.g. wheat straw and corn stover), where autohydrolysis often is enough, and softwood (e.g. spruce) where an acid, such as SO_2 or H_2SO_4, typically needs to be added due to the more recalcitrant material and lower degree of acetylation (Alvira et al. 2010; Kumar et al. 2009).

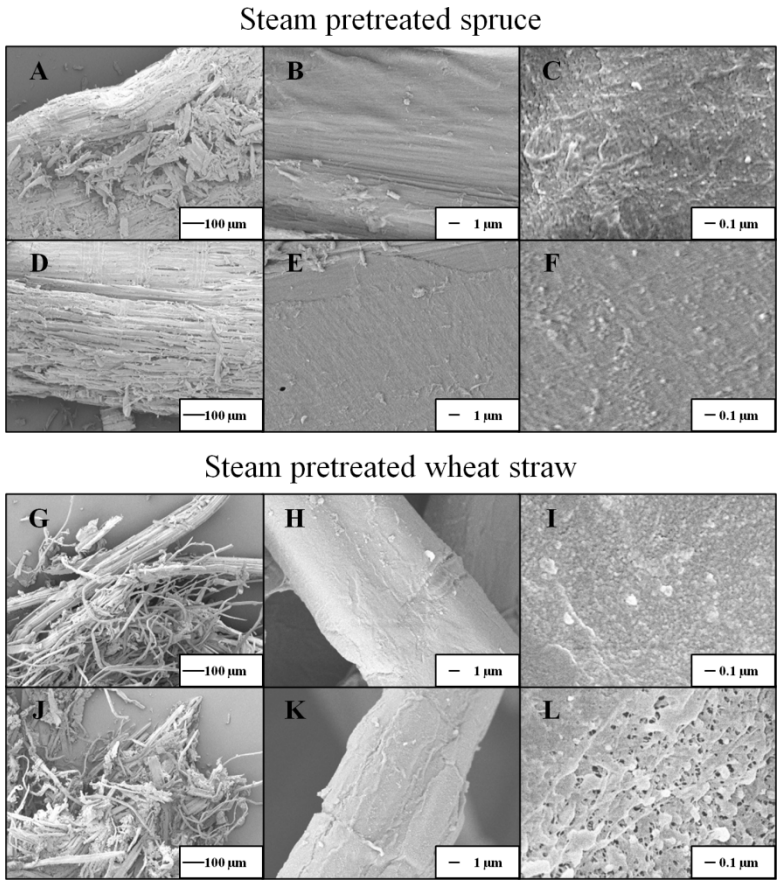

Figure 2.3. SEM (scanning electron microscopy) images of differently steam pretreated spruce and wheat straw. The pretreatment conditions were (A-C): 5 min, 210 ˚C and 2.5 % (w/w) SO_2, (D-F): 10 min, 190 ˚C and 2.5 % (w/w) SO_2, (G-I): 2 min, 190 ˚C and 0.2 % H_2SO_4, and (J-L): 10 min, 210 ˚C and 0.2 % H_2SO_4. Adapted from Piccolo et al. (2010)

Since steam pretreatment typically results in rather high sugar recovery and requires relatively low capital investment, it is considered one of the most promising methods for industrial implementation (Alvira et al. 2010; Chandra et al. 2007). Furthermore, STEX has been implemented at several pilot/demonstration scale plants, such as DOE's pilot plant[3] in Golden, Colorado (US), EPAB's demonstration plant[4] operated by SEKAB in Örnsköldsvik, Sweden, Iogen's demonstration plant[5] in Ottawa, Canada, and Inbicon's demonstration plant in Kalundborg, Denmark (Larsen et al. 2012).

Inhibitor formation during STEX

If too severe a pretreatment is used, i.e. too long residence time, too high temperature or too high acid concentrations, part of the formed monomeric sugars will be further degraded into aldehydes and organic acids (Figure 2.4). This does not only create a yield loss, but can also create severe problems for the enzymatic hydrolysis and especially fermentation, since many biomass degradation products have been shown to be highly inhibitory for most fermenting microorganisms (Almeida et al. 2007; Almeida et al. 2011; Palmqvist and Hahn-Hagerdal 2000). This is further discussed in Chapter 2.2.3.

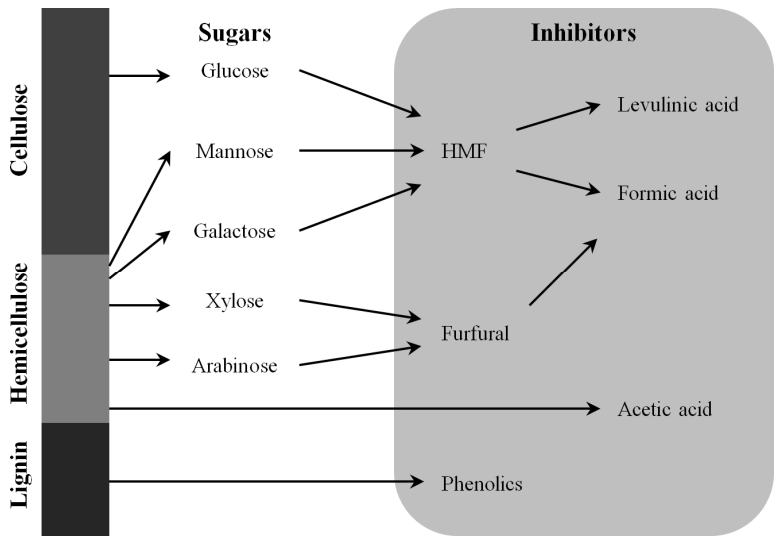

Figure 2.4. Common inhibitors generated during pretreatment of lignocellulosic material. Adapted from Almeida et al. (2007).

[3] http://www.nrel.gov/docs/fy00osti/28397.pdf (2014-03-27)

[4] http://www.sekab.com/biorefinery/demo-plant (2014-03-27)

[5] http://www.iogen.ca/technology/demo-plant.html (2014-03-27)

The majority of the inhibitors can be divided into furans, phenolics and weak acids. Amongst the furans, the two main inhibitors are 2-furaldehyde (or furfural) and 5-hydroxymethyl-2-furaldehyde (or HMF). These are degradation products of C5 and C6 sugars, respectively. Under harsh pretreatment conditions, the furans can be further degraded into both levulinic and formic acid. The phenolic compounds are degradation products of lignin. Acetic acid on the other hand is different in that it is inherent in the material in terms of acetyl groups on the hemicellulose backbone. The acetyl groups are released during pretreatment and hence the amount of acetic acid present in the final pretreated slurry is less determined by the pretreatment method itself, but rather by the composition of the raw material.

2.2.2 Enzymatic hydrolysis

After pretreatment, the main part of the cellulose still remains in polymeric form, and depending on the pretreatment, some of the hemicelluloses may also remain in polymeric or oligomeric form. To release the reminder of the sugars, a set of enzymes, mainly cellulases, are needed. For a long time, enzymatic hydrolysis was regarded as the primary bottleneck in the production of bioethanol from lingocellulose (Lynd et al. 2008). This was mainly due to the slow action of the cellulase mixtures and the need for large amounts of expensive enzymes. However, impressive research efforts during the past decades have resulted in both reduced enzyme loadings and lower production costs, significantly contributing to the present commercialization. Part of this progress was due to the awarding of a major DOE research grant (DE-PS36-07GO97034) to several major enzyme suppliers with the goal of cutting production cost of enzymes down to US$0.12 per gallon of produced ethanol by 2012. Precursors of theses enzyme cocktails were bench-marked by the National Renewable Energy Laboratory (NREL) as part of the funding agreement (McMillan et al. 2011).

The basic cellulase cocktail

The fungal families *Trichoderma* and *Aspergillus* are typically used for industrial cellulase production due to their high secretion levels (Zhang and Lynd 2004). Their cellulase systems comprises three main classes of enzymes, i.e. endo-1,4-β-glucanases (EG), exo-1,4-β-glucanases (also called cellobiohydrolases, CBH) and β-glucosidases (BG), and the enzymatic hydrolysis of cellulose is regarded as a synergistic action between these enzymes classes (Van Dyk and Pletschke 2012). *Trichoderma reesei* excretes at least 2 different cellobiohydrolases (CBH1-2), five endoglucanases (EG1-5), β-glucosidases and hemicellulases (Vinzant et al. 2001). The main enzymes are CBH1-2 and EG1-2, and these constitute more than 90% of the total excreted cellulases (Zhang and Lynd 2004).

The CBH works in a processive manner, cutting cellobiose units from either the reducing (CBH1) or the non-reducing (CBH2) end of the cellulose chain by hydrolyzing the β-1,4-glycosidic bond. A typical CBH enzyme consists of 3 different domains – a cellulose-binding module (CBM) to find and attach to the substrate, a hydrolytic domain containing the active site and finally a linker giving the enzyme its flexibility. The CBM of CBH1 (likely the most studied cellulase enzyme) binds the enzyme to the cellulose chain in order to keep the catalytic domain close to its substrate. It has also been found that CBMs help to break the intermolecular bond of the cellulose, disrupting its crystalline structure and making it more accessible to the hydrolytic domain (Hall et al. 2011a). Furthermore, CBMs have a thermostabilizing function (Hall et al. 2011b). Despite the many positive attributes of CBMs, recent studies suggest that when working at high substrate concentrations, which typically is not the case in mechanistic studies, CBMs are not needed. This is likely due to the close proximity to the substrate and the lack of free water in the system (Várnai et al. 2013). Cellulases with removed CBMs even outperformed their corresponding CBM containing cellulases at high substrate concentration (Le Costaouëc et al. 2013). Furthermore, enzymes without a CBM could potentially be easier to recycle (Pakarinen et al. 2014). The active site is located in a "tunnel" within the hydrolytic domain of CBH. This allows for a processive action, by passing the cellulose chain through the tunnel, while progressively cleaving one cellobiose unit at a time. However, due to this tunnel, CBHs have a low desorption ability, which significantly reduces the processivity in lignocellulosic substrates when there is steric hindrance, caused by the material structure or other enzymes (Jalak and Väljamäe 2010; Igarashi et al. 2011).

Endo-1,4-β-glucanases (EG) do not work in the same processive manner along the cellulose chain as the CBH. Instead, they bind randomly to the cellulose polymer and hydrolyze an internal β-1,4-glycosidic bond in the glucan chain (preferably in amorphous regions). This effectively reduces the degree of polymerization of the cellulose chain and creates two new chain-ends for the CBH to act on.

Strictly speaking, β-glucosidases (BG) are not cellulases since their main substrate is cellobiose, which is released from the cellulose by CBHs. The β-glucosidases catalyze the hydrolytic splitting of cellobiose into two glucose molecules.

The synergistic action between these three enzymes is well studied and very important, especially since the enzymes are end-product inhibited by cellobiose in particular. Due to this strong inhibition, it is crucial to have an enzyme cocktail with sufficiently high BG activity in order for the hydrolysis to proceed even when the enzymatic activity declines due to high sugar concentrations. BGs are in turn end-product inhibited by high glucose concentrations.

The introduction of a new star player – LPMO

A major breakthrough for efficient hydrolysis of recalcitrant biomass came recently when a new type of enzyme and enzyme action was identified. The enzyme, at discovery, was classified as a glycoside hydrolase, belonging to the GH61 family (Harris et al. 2010). The enzyme has now been reclassified as a lytic polysaccharide monooxygenase (LPMO), since it possesses an oxidizing rather than hydrolytic action and now belongs to the AA9 family (Levasseur et al. 2013). This enzyme cleaves intermolecular bonds in the glucan chain by oxidizing the C1/C4 carbon. This enzyme class thus has a completely different way of creating new reaction sites for the CBH (Horn et al. 2012; Dimarogona et al. 2013). Since it appears that LPMOs do not need a specific binding site on the cellulose it has the potential to break crystalline cellulose structures, hence creating a more reactive substrate for the other previously studied cellulases (Horn et al. 2012; Dimarogona et al. 2013). In order for the LPMOs to function properly, a substance acting as an electron donor is needed. This could either be added externally (e.g. ascorbic acid or glutathione) or be an enzyme working synergistically with LPMOs, such as cellobiose dehydrogenase (CDH) (Dimarogona et al. 2013; Horn et al. 2012). CDH oxidizes cellobiose and is excreted naturally by for example wood degrading fungi (Zamocky et al. 2006). Recent studies further indicate that lignin can function as the electron donor when degrading lignocellulosic materials (Cannella et al. 2012; Dimarogona et al. 2012). The identification and addition of LPMOs have contributed greatly to the remarkable progress of commercial enzyme blends (Cannella et al. 2012). A schematic picture of the synergistic action of cellulases, including LPMOs, can be seen in Figure 2.5.

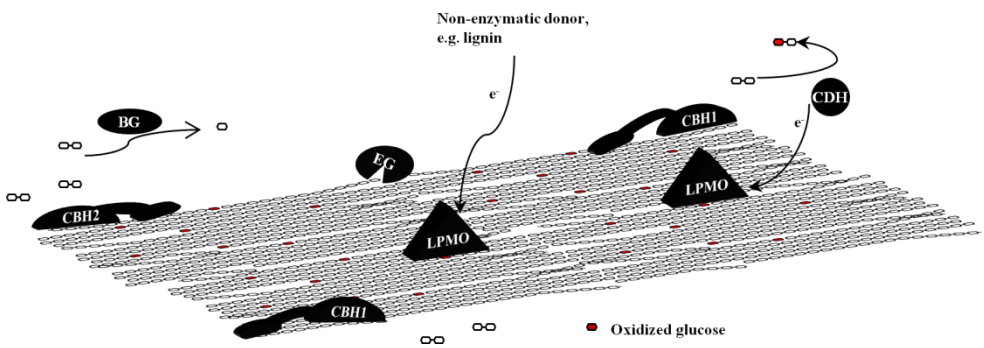

Figure 2.5. Enzymatic degradation of cellulose. Adapted from Horn et al. (2012).

Hemicellulases, auxiliary enzymes, and synergism – a more complex picture

Working with lignocellulosic substrates, rather than pure cellulose, complicates the enzymatic hydrolysis and usually calls for supplementations with other enzyme types in order to effectively hydrolyze the material. Since there is a trend

towards milder pretreatment methods, where the major fraction of the hemicellulose is not degraded to monomers but rather remains in oligomeric form, xylan or xylo-oligo degrading enzymes have become more important. It has been shown that both xylobiose and larger xylo-oligomers exhibit a very strong inhibition on the activity of cellulases (Kont et al. 2013; Qing and Wyman 2011). Research has also been devoted to synergism studies between hemicellulases and different auxiliary enzymes (Van Dyk and Pletschke 2012). Synergistic effects have for example been found between xylanase and acetyl xylan esterase (Kam et al. 2005; Selig et al. 2008), α-L-arabinofuranosidase and endo-xylanase (Shi et al. 2010) and α-galactosidase and β-mannanase (Wang et al. 2010).

2.2.3 Fermentation

Baker's yeast, *Saccharomyces cerevisiae*, is one of the most commonly used microorganism in sugar- and starch-based ethanol production today and will likely play an important role in lignocellulosic biorefineries as well.

There are of course many other potential candidates for ethanol production, including the yeasts *Scheffersomyces stipitis*, *Kluyveromyces marxianus*, *Dekkera bruxellensis* (Blomqvist et al. 2010) and the bacteria *Zymomonas mobilis* (Rogers et al. 1982) and *Escherichia coli*, reviewed by among others Jarboe et al. (2007), all with different pros and cons with respect to for example ethanol tolerance, productivity and yield. The focus in this dissertation will, however, be on *S. cerevisiae*. The xylose fermenting strain TMB3400 (Wahlbom et al. 2003) has been used in all experimental work carried out.

Some of the main advantages of *S. cerevisiae* as an ethanol producer are its well-documented use in industry, its well-established process technology for large scale production (Ostergaard et al. 2000) and its GRAS (Generally Regarded As Safe) status. Moreover, it has a very high ethanol tolerance (Casey and Ingledew 1986; Verduyn et al. 1990) and has been shown to be relatively robust towards many of the inhibitors present in pretreated lignocellulosic hydrolysates (Hahn-Hägerdal et al. 1994; Olsson and Hahn-Hägerdal 1993). Lastly, *S. cerevisiae* is one of the most studied and well-characterized microorganisms with a full range of genetic engineering tools available (Nevoigt 2008).

S. cerevisiae is chemoheterotrophic, i.e. it uses organic material both as building blocks and as energy source. Furthermore, *S. cerevisiae* is a facultative anaerob, meaning that it can grow under both aerobic and anaerobic conditions (Visser et al. 1990). Fermentation of the hexose sugars glucose and mannose is generally very effective in *S. cerevisiae*, while galactose fermentation is strain specific (Lindén et al. 1992). However, fermentation of pentose sugars does not take place in wild-type *S. cerevisiae*. Given the importance of xylose fermentation for producing ethanol from, for example, agricultural residues, grasses and hardwoods

(see Table 2.1), large efforts have been made in engineering of *S. cerevisiae* to introduce xylose fermentation capabilities (as discussed later on this dissertation).

The metabolism of S. cerevisiae

The first step in the metabolism is to transport the substrate (mainly glucose) into the cell. In *S. cerevisiae*, sugar transport is achieved through facilitated diffusion. There are at least 20 different transport proteins, including the Hxt-transporters, which are able to transport hexoses through the membrane of this yeast (Boles and Hollenberg 1997). The different transport proteins exhibit different affinity to glucose and in order to save energy the cell expresses only the proteins needed for the actual sugar concentrations present, i.e. at low glucose concentrations, high affinity transporters are expressed and at high glucose concentrations low affinity transporters are expressed (Özcan and Johnston 1999).

Once inside the cell, glucose is phosphorylated by hexokinase to glucose-6-phosphate. Mannose is also phosphorylated by hexokinase and further isomerized by mannose-6-phosphate isomerase to fructose-6-phosphate (Zimmermann and Entian 1997). Galactose on the other hand is converted to glucose-6-phosphate through the Leloir pathway before entering the glycolysis (Timson 2007). In the glycolysis, glucose-6-phosphate and fructose-6-phosphate is converted to pyruvate in a series of reactions, resulting in a net formation of 2 ATP (adenosine triphosphate) and 2 NADH (nicotinamide adenine dinucleotide) per sugar molecule (Figure 2.6). In a fully respiratory metabolism, pyruvate is decarboxylated, via the pyruvate dehydrogenase, and the resulting Acetyl-CoA is subsequently oxidized to carbon dioxide in the tricarboxylic acid (TCA) cycle. The formed redox-cofactors, four moles of NADH and one mole of Flavin adenine dinucleotide ($FADH_2$) per mole of pyruvate, are then reoxidized to NAD+ and FAD in the oxidative phosphorylation, yielding ATP. During fermentation, which necessarily takes place under anaerobic conditions, pyruvate is instead decarboxylated to acetaldehyde via the pyruvate decarboxylase. Acetaldehyde is further reduced to ethanol by alcohol dehydrogenase, consuming one mole of NADH. This means that the complete conversion of one mole of glucose to two moles of ethanol is redox neutral. The fermentative pathways can be active also at aerobic conditions, despite that respiration generates more ATP. This is known as respiro-fermentative growth, or the crabtree effect, and originates in an overflow at the pyruvate node caused by high growth rate and a limited respiratory capacity (Kappeli 1986). This overflow metabolism becomes highly important during yeast propagation, since ethanol formation in this case constitutes a biomass yield loss. Design of fed-batch cultivation, where the glucose uptake rate can be controlled, is thus important.

The maximum yield of ethanol from glucose during fermentation is 2 moles / mole (or 0.511 gram / gram). However, glycerol is (sometimes) also produced under anaerobic conditions. This is a way to regenerate NAD+, needed for biosynthetic

reactions (van Dijken and Scheffers 1986). Furthermore, glycerol can be produced as a response to osmotic stress (Nevoigt and Stahl 1997).

The pentose phosphate pathway (PPP) is another metabolic route in the carbon metabolism. It is needed to provide carbon precursors (including pentose-phosphates) as well as NADPH for the various biosynthetic (anabolic) reactions. Moreover, when genetically engineered to consume xylose, the yeast utilizes the PPP as a starting point for the xylose metabolism. The central carbon metabolism of *S. cerevisiae* during fermentation is shown in Figure 2.6.

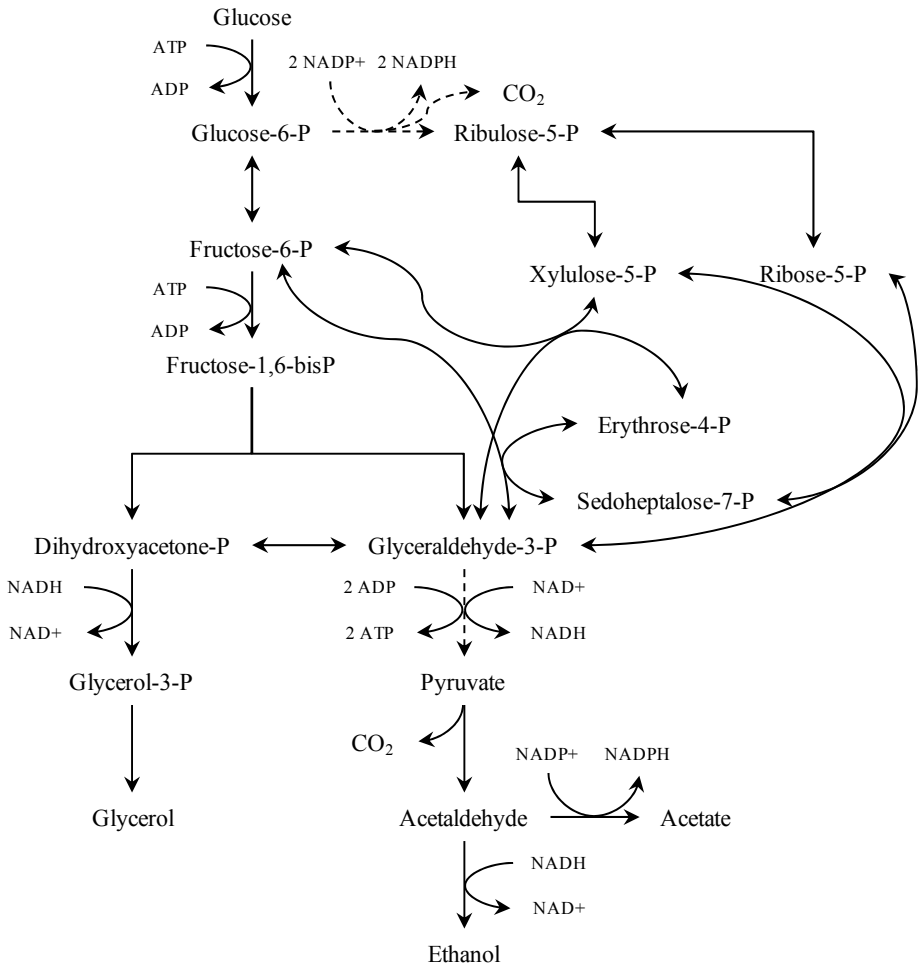

Figure 2.6. The central carbon metabolism of *S. cerevisiae* during fermentation.

Xylose utilization in S. cerevisiae

All sugars, present in significant amounts, should preferably be fermented to achieve maximum ethanol yield and concentration when working with lignocellulosic substrates (Sassner et al. 2008). This usually means that, apart from glucose, at least mannose (softwoods) and xylose (agricultural residues, grasses and hardwood) should be fermented. *S. cerevisiae* naturally ferments mannose, which is an advantage of using softwood (e.g. spruce) as a substrate for bioethanol production. However, there is no fermentation capacity for xylose in native strains of *S. cerevisiae*. Since xylose is the second most dominant sugar found in most plant materials, genetic and metabolic engineering has been very focused on introducing efficient xylose utilizing pathways in *S. cerevisiae* (reviewed by, for example, Almeida et al., (2011); Van Vleet and Jeffries, (2009), Matsushika et al., (2009), Hahn-Hägerdal et al., (2007)). In order to introduce an efficient xylose fermenting capacity, one of the two main pathways needs to be introduced (Figure 2.7). The options are i) the two step conversion of xylose to xylitol and further on to xylulose, which is catalyzed by xylose reductase (XR) and xylitol dehydrogenase (XDH) respectively, or ii) the one step isomerization of xylose to xylulose catalyzed by xylose isomerase (XI). The engineered strains are commonly referred to as XR/XDH, in the former case, or XI strains in the latter case. In both cases, xylulose is then phosphorylated by xylulokinase (XK) before entering the main glycolysis through the PPP (Figure 2.6).

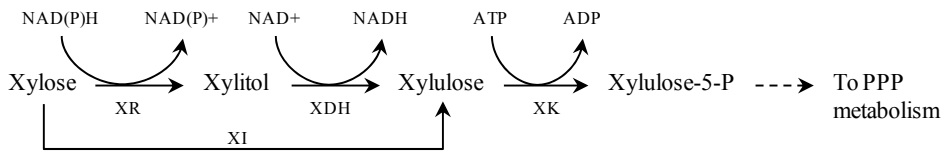

Figure 2.7. The XR/XDH and the XI pathways. XI: xylose isomerase, XR: xylose reductase, XDH: xylitol dehydrogenase, XK: xylulokinase.

Only introducing the pathways converting xylose to xylulose has, however, been shown to be insufficient. In order to reach efficient conversions a number of additional modifications have been introduced, including over-expression of enzymes in the PPP (Karhumaa et al. 2005) and insertion/up-regulation of different xylose transporters (Fonseca et al. 2011; Runquist et al. 2009; Runquist et al. 2010). Moreover, both pathway options have their specific drawbacks. For the XR/XDH strains, the main issue has been the difference in co-factor dependencies for the two reactions, where XR preferably uses NADPH and XDH typically uses NAD+. This leads to a co-factor imbalance and consequently xylitol excretion. With the XI strain this imbalance is avoided, and instead the main challenge has been to express an active XI enzyme which works effectively at the low temperatures typically used for yeast fermentation. This was not achieved until about a decade ago (Kuyper et al. 2003; Kuyper et al. 2005).

After introduction of the necessary genes to enable xylose utilization, evolutionary engineering, preferably in xylose rich media, has been found advantageous to 'fine tune' the regulatory functions for optimal performance through spontaneous mutation (Lee et al. 2012; Zhou et al. 2012).

Inhibition from hydrolysate

Fermenting pure sugar solutions does not present any particular challenge for most considered microorganisms. However, pure sugar solutions do not exist in a lignocellulosic biorefinery. Instead, the sugar solution contains a variety of biomass degradation products, produced during the pretreatment of the material (Figure 2.4). Many of them have been shown to negatively impact the fermenting microorganism (Klinke et al. 2004; Palmqvist and Hahn-Hagerdal 2000). Hence, the challenge is to maintain a high fermentation capacity and productivity under the influence of these different inhibitors. The inhibition problem obviously gets bigger when working with non-diluted, concentrated slurries. If an inhibitor removal step is not desired these problems needs to be dealt with through a combination of strain robustness and process design concepts (Almeida et al. 2007).

As mentioned previously in section 2.2.1 acetic acid is formed during pretreatment due to deacetylation of the material's inherent acetyl groups bound to the hemicellulose backbone. Acetic acid will therefore be present as an inhibitor regardless of pretreatment as long as the whole pretreatment slurry is used (i.e. when no solid-liquid separation or detoxification step is included). As a weak acid (pKa value of 4.76), the main form in which acetic acid will be present is highly dependent on the pH, with large changes in the typical fermentation pH region of about 5. This can strongly affect the yeast, since it has been shown that it is only the un-dissociated form of the acid that causes inhibition by diffusing across the cells' plasma membrane to the interior (Casal et al. 1996). Inside the cell, where the pH is higher, the hydrogen proton dissociates and in order to keep the intracellular pH the proton needs to be exported from the cell at the cost of ATP.

The inhibitory effect of acetic acid at different pH levels has been demonstrated previously for *S. cerevisiae* in defined media, both when growing on pure glucose (Taherzadeh et al. 1997) or xylose (Bellissimi et al. 2009) and during co-consumption of glucose and xylose (Casey et al. 2010). The inhibitory effect seems more detrimental for xylose consumption and its effect on process design will be further discussed in chapter 4.3.

2.2.4 Simultaneous or separate hydrolysis and fermentation?

Already in the 1970s, the idea of performing enzymatic hydrolysis together with the fermentation was introduced by Gauss et al. (1976). The term 'Simultaneous Saccharification and Fermentation' (SSF) quickly became the common notation for the process, even though the authors themselves did not introduce this term. Since then, SSF has been compared to 'Separate Hydrolysis and Fermentation' (SHF) as the two main process options for lignocellulosic conversion to ethanol. Both have their principal benefits and drawbacks, and the question of whether SHF or SSF is preferable will depend on for example which feedstock, yeast and enzymes that are used. The major advantage of the SHF concept is that the enzymatic hydrolysis and fermentation can be optimized separately, mainly with respect to temperature. The enzymes typically have their optimum around 45-55 ˚C, whereas *S. cerevisiae* typically grows best at 30-35 ˚C. The temperature tolerance is rather strain-dependent though, and some strains are able to ferment at as high temperature as 40 ˚C (Zhang et al. 2012). The end-product inhibition of the enzymes has traditionally been regarded as the major drawback of SHF. Recently however, this may be starting to change due to the progress in enzyme cocktail development (Cannella and Jørgensen 2014; McMillan et al. 2011). An SSF process will require a temperature compromise between the optimum temperature of the enzymatic hydrolysis and that of the fermentation, often favoring the fermentation. The main benefits, on the other hand, are that end-product inhibition of the enzyme can be avoided by the continuous fermentation of the sugars to ethanol and that fewer reactors can be used. More elaborate process designs based on SHF and SSF will be discussed in chapter 4.

2.3 The transition to commercial scale production

The lignocellulosic ethanol industry is now moving from pilot scale to demonstration/full scale operation. Several production facilities are under construction worldwide (Table 2.3), as highlighted by for example Balan et al. (2013) and Janssen et al. (2013). Despite the huge market domination of Brazil and the US on first generation bioethanol, some of the first pilot and small scale demonstration plants are actually located in Europe, particularly in the Nordic countries. For example, the "Biorefinery Demo Plant" in Örnsköldsvik, Sweden, and the Danish Inbicon demonstration plant outside of Kalundborg (Larsen et al. 2012). Furthermore, the two largest lignocellulosic ethanol producers are currently located in Europe – the Borregaard biorefinery in Norway, where ethanol is a co-product of lignosulfonates and cellulose fibers production, and the first dedicated large scale lignocellulosic ethanol plant recently built in Crescentino, Italy, by Beta Renewables.

Table 2.3. Compilation of larger demostration plants (capacity of more than 1 000 annual tonnes of ethanol) in operation and commercial plants under construction/in operation according to IEA Task 39 database[6].

Company	Location	Feedstock	Capacity
Operational demonstration plants			
Abengoa Biorefinery	Salamanca, Spain	Cereal straw	4 000 annual tonnes
Inbicon (DONG Energy)	Kalundborg, Denmark	Wheat straw	4 300 annual tonnes
Iogen Corporation	Ottawa, Canada	Agricultural residues	1 600 annual tonnes
Operational commercial plants			
Beta Renewables	Crescentino, Italy	Arundo donax, wheat straw	60 000 annual tonnes
Borregaard Industries AS	Sarpsborg, Norway	Spent sulphite liquor	15 800 annual tonnes
Commercial plants under construction			
Abengoa Bioenergy	Hugoton, United States	Corn stover, wheat straw, swithgrass	75 000 annual tonnes
POET-DSM Advanced Biofuels	Emmetsburg, United States	Agricultural residues	75 000 annual tonnes

Several commercial plants are currently being constructed in the US and more projects are planned around the world (Balan et al. 2013; Janssen et al. 2013). However, activities in Europe are low. Probable reasons for this include the greater availability of cheaper feedstock in the US and difference in political incentives between the US and Europe. Uncertainties in future policies, and within the EU's regulatory framework, could drive companies with demonstration units in Europe to deploy their technology elsewhere (Balan et al. 2013). This has for example been the case for Abengoa, which is currently constructing a commercial plant in Hugoton, US, or Beta Renewables which is now partnering with Graalbio in Brazil (Balan et al. 2013).

[6] http://demoplants.bioenergy2020.eu/ (2014-03-19)

Chapter 3

High solids enzymatic hydrolysis

Operating a biorefinery at a high solids loading could potentially offer advantages from a feasibility/economical point of view. Numerous techno-economical reports argue that high solid loadings have a positive effect on many dimensions of process economy. For example, a higher ethanol concentration gives reduced distillation costs (Galbe et al. 2007), and less use of process water reduces wastewater treatment cost as well as process energy needs (Wingren et al. 2003). The cost of equipment also goes down when operating at a high solids loading (Humbird et al. 2010; Macrelli et al. 2012). These benefits, coupled with the fact that commercial scale facilities are now being constructed, has meant that, in the last few years, research has intensified towards solving the various issues created by working with highly concentrated fiber suspensions. In this chapter, which is based on **Paper I-V**, a description of the complex rheology of pretreated biomass is presented, and discussed in relation to mixing at industrially relevant solid loadings.

Before diving into the work carried out within this dissertation, the term 'high solid' needs to be addressed since it is an ambiguous one. 'High solids loading' has different meanings for different people. Furthermore, it probably differs between materials. As will be shown later on in this chapter, different materials behave rather different at similar solids content. One could imagine defining 'high solid' as the amount of solids that would be needed to (theoretically) reach a specific ethanol target, such as 4 or 5 wt-%, which is commonly regarded as a feasible ethanol concentration from a distillation point of view (Galbe et al. 2007). This definition would, however, be highly material specific, depend on whether pentoses are fermented or not and if washed or unwashed pretreated slurry is used. This leads to the second word which could cause confusion, i.e. the word 'solids'. This is not as straight forward as one may first imagine, since unwashed pretreated biomass slurries contain both water insoluble solids (WIS) and soluble solids (e.g. sugars and short oligosaccharides). The combination of soluble solids and the WIS constitutes the dry-matter (DM) of the material. In this dissertation, a high solids loading refers – in a pragmatic way – to a WIS loading of at least 10 wt%.

3.1 Rheology of pretreated lignocellulosic material

The term 'rheology' was introduced by the Chemistry professor Eugene C. Bingham in the 1920s and is defined as *the study of the deformation and flow of matter*. To what extent a fluid flows, or deforms, under the influence of an external force is described by its viscosity. The viscosity is a measure of a fluid's inner resistance to flow – or shear – and is determined by relating the velocity gradient in the fluid to the applied shear force. Fluids are typically classified as being either Newtonian or non-Newtonian, depending upon if their viscosity changes with the applied shear rate or not. Some fluids also exhibit a yield stress. This means that below a certain amount of applied shear stress the fluid does not flow, rather it simply deforms plastically like a solid. Once the yield stress is exceeded, it starts to behave as a viscous fluid. A number of well-established viscosity models are used to describe non-Newtonian flow behavior, for example the Bingham model to describe yield stress behavior, power law models to describe shear thinning and dilatant behavior, or the Herschel-Bulkley model, which combines yield stress with shear-thinning behavior.

During pretreatment of lignocellulosic biomass, part of the material is dissolved, creating a suspension of fiber particles – a slurry. Fiber suspensions in general are often found to be shear thinning fluids, i.e. their viscosity decreases with increased shearing (Bayod et al. 2007; Buscall et al. 1987; Luckham and Ukeje 1999; Ouden and Vliet 1997; Zhou et al. 1999). Most rheological studies on pretreated biomass have been carried out on corn stover in the US. Considerable knowledge has accumulated on the behavior of this material, including the rapid increase of viscosity with increased fiber concentration (Knutsen and Liberatore 2009; Pimenova and Hanley 2004; Roche et al. 2009a; Viamajala et al. 2009). A wide range of other materials have also been characterized, for example wheat straw, red oak saw dust, barley straw and also pure cellulose suspensions (Dasari and Berson 2007; Rosgaard et al. 2007; Skovgaard et al. 2014; Tozzi et al. 2014). However, none of them have been as extensively studied as corn stover.

Measuring the rheological properties of pretreated biomass, however, is not trivial, with some of the main concerns being wall slip and particle settling (Stickel et al. 2009). For the work carried out within this dissertation, a rotational rheometer with a rotating vane was used (Figure 3.1). This has been suggested as the preferred method for biomass characterization (Knutsen and Liberatore 2009) and is furthermore known to minimize slip problems (Barnes 1995; Barnes and Nguyen 2001). Other more applied methods have also been reported, such as the use of torque measurements in a reactor equipped with a helical impeller (Pimenova and Hanley 2003). For a more detailed description of measurements, see the material and method section in **Paper I**.

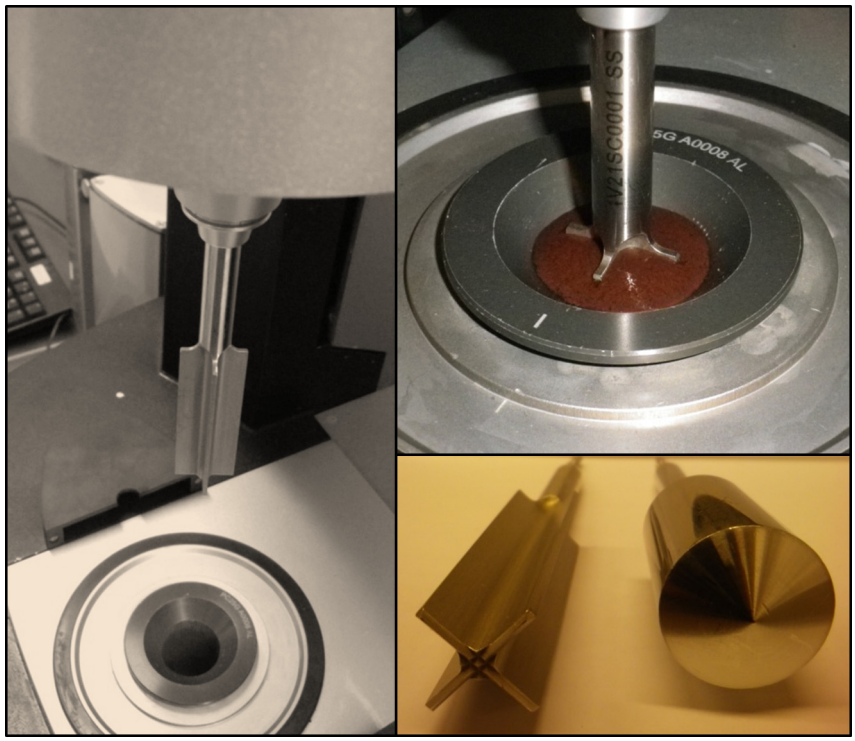

Figure 3.1. The rheometer used for rheological characterization in **Paper V** (a similar set-up was used in **Paper I**). The bottom right picture shows the common cylinder (right) often used for rheological measurements and the vane (left) which is usually preferred when measuring fiber suspensions to avoid slip effects.

Due to the complexity of pretreated biomass, it is crucial to understand the rheology – and the changes in rheology during processing – to effectively design processes and equipment (Viamajala et al. 2010), especially in a full scale plant. In **Paper I**, the first (to our knowledge) comprehensive rheological characterization of steam pretreated spruce is presented. Both the viscoelastic properties of the material, and the flow behavior were investigated in relation to both WIS concentration and particle size distribution (PSD). Flow curve measurements were furthermore carried out on a differently pretreated spruce material in both **Paper IV** and **Paper V**.

3.1.1 Rheological properties of steam pretreated spruce

Pretreated spruce was found to be strongly shear-thinning, meaning the viscosity (μ) of the fluid decreases dramatically as the applied shearing rate (γ) increases. The shear-thinning behavior was well-captured by the power-law viscosity model:

$$\mu_{app} = K_{PL} \cdot \gamma^{n-1} \; (Eq. 3.1)$$

Where μ_{app} is the apparent viscosity (Pa s) and γ the shear rate (s^{-1}), K_{PL} is the consistency index (Pa s^n) and the dimensionless n is the flow behavior index. As expected, a strong influence of WIS loading was found on the viscosity (Figure 3.2A). The rather large difference in viscosity between the two different sets presented in Figure 3.2 arose from milling the pretreated material. The fact that milling increased the viscosity was not in accordance with previous studies on, for example, red-oak saw dust (Dasari and Berson 2007) or pretreated corn stover (Ehrhardt et al. 2010; Viamajala et al. 2009), where a lower viscosity was found for smaller particle sizes. This latter behavior is typically true for mono-disperse systems, i.e. systems containing only one particle size or a very narrow PSD. For systems containing a multitude of differently sized particles, on the other hand, a narrower PSD has been found to increase viscosity due to the creation of more junction points in the fiber network (Bayod et al. 2007; Ouden and Vliet 1997). The strength of a fiber network is determined by the junction points of the fibers. When the PSD is broad, one can imagine the larger particles forming the matrix, which provides the strength of the network. Smaller particles are trapped within the void of this matrix and hence do not contribute to its strength. If the PSD becomes narrower, a larger fraction of the available fibers are able to contribute to the strength of network. This explanation seems very likely for the results presented here, since it was shown that the main effect of milling was a reduction in size of the largest particles (Figure 3.2B).

A qualitatively similar flow behavior was also found for the pretreated spruce used in **Paper V**. However, the absolute value of the viscosity was markedly lower (Figure 3.3A). The materials used in **Paper I** and **Paper V** were both spruce, steam pretreated at similar residence times and temperature, and with SO_2 as a catalyst. The difference, however, was in the pretreatment equipment used. Whereas the material from **Paper I** was pretreated in batch mode at bench scale level (about 0.75 kg DM per batch), the material in **Paper V** was pretreated in continuous mode at demonstration scale level (50 kg DM per hour). One of the main differences between these pretreatments is the mechanical forces exerted on the material in the continuous reactor, which effectively created a broad particle size distribution with a significantly smaller mean particle size (Figure 3.3B). The mean particle size of the continuously pretreated spruce materials was about 60 % of that of the batch pretreated material (15μm vs. 25μm).

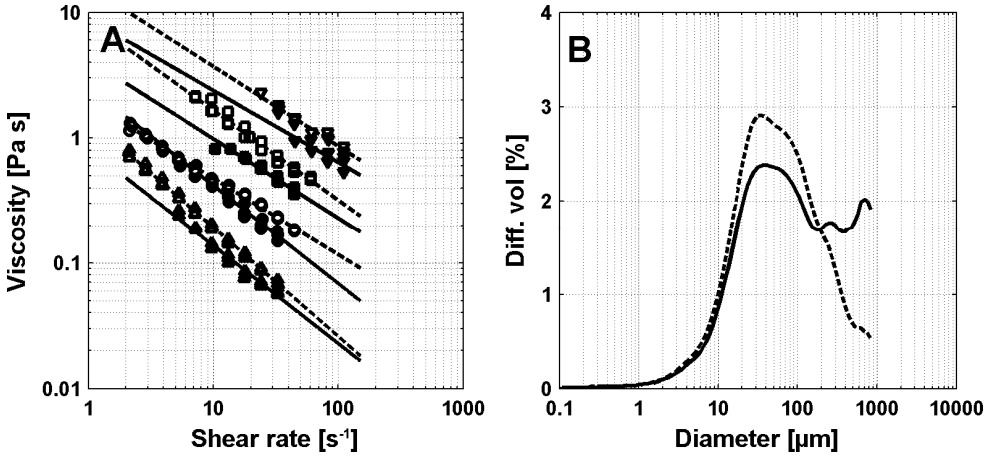

Figure 3.2. Viscosity (A) and PSD (B) of batch steam pretreated spruce at 6 % WIS (▲), 8 % WIS (●), 10 % WIS (■) and 12 % WIS (▼). Open symbols and dotted lines represents milled material. Solid symbols/lines represent the original material. (Data from **Paper I**)

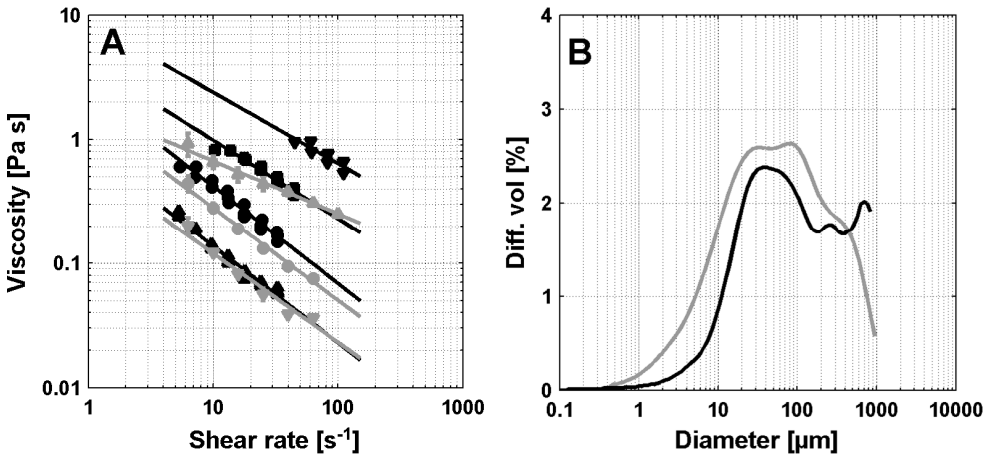

Figure 3.3. Comparison of viscosity (A) and PSD (B) between differently pretreated spruce materials. Black represents batch pretreated material at 6 % WIS (▲), 8 % WIS (●), 10 % WIS (■) and 12 % WIS (▼). Grey represents continuously pretreated material at 10 %WIS (▼),13.5 % WIS (■) and 17 % WIS (▲). (Data from **Paper I** and **Paper V**)

Yield stress and elastic modulus of steam pretreated spruce

In addition to flow curve measurements, yield stress, another important rheological property, was also measured in **Paper I**. The physical meaning of yield stress is that it is the shear stress at which the fluid changes from elastic to viscous behavior. Phrased differently, below the yield stress the sample will deform elastically (like stretching a spring) and above the yield stress the sample will flow like a liquid. Characterizing the yield stress is of great importance since

fluids exhibiting a high yield stress can cause severe problems during mixing in stirred tank reactors, as further discussed in chapter 3.2.

Two different types of yield stress can be defined, i.e. static and dynamic (Nguyen and Boger 1992). Direct measurements, like the oscillatory stress sweep presented in **Paper I**, give a value of the static yield stress of the material. Indirect estimations of yield stress, typically by extrapolation of flow curve measurements, instead give the dynamic yield stress. A strong dependence on WIS concentration was found for the yield stress (Figure 3.4), in accordance with previously published results on corn stover (Ehrhardt et al. 2010; Viamajala et al. 2009). In addition, an influence of PSD was found where the milled material, i.e. a narrower PSD, resulted in a significantly stronger fiber network and hence higher yield stress at corresponding WIS concentration.

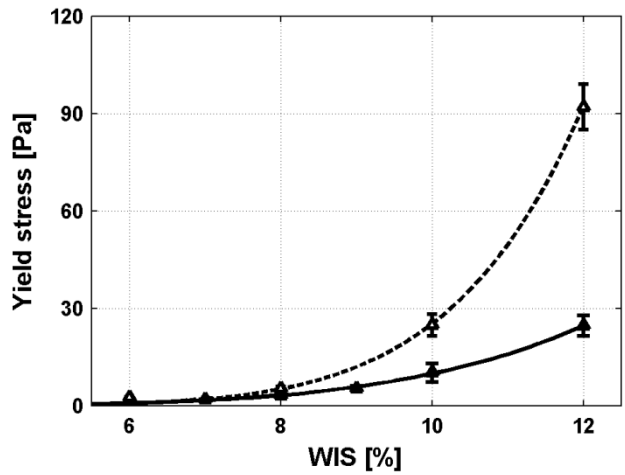

Figure 3.4. Yield stress as a function of WIS content for batch pretreated spruce material. Solid symbols/line represents the original material. Open symbols and dotted line represents milled material. (Data from **Paper I**)

Rheological changes during enzymatic hydrolysis

During enzymatic hydrolysis, part of the solid material is hydrolyzed into soluble sugars, resulting in both a reduction in WIS concentration and a change in chemical composition, as well as structure, of the fibers. Not surprisingly, the viscosity decreases during enzymatic hydrolysis (Figure 3.5). However, the decrease cannot simply be estimated based on the remaining WIS content. The change in rheological properties during enzymatic hydrolysis was especially pronounced for the yield stress. For pretreated spruce, an approximately 10-fold decrease in yield stress was found at 40 % conversion, compared to less than a 4-fold decrease in viscosity (**Paper I**). The rheological changes during hydrolysis vary significantly between raw materials, however, and Roche et al. (2009a) found

a 100-fold decrease in yield stress for corn stover (20 % initial solids content) at about 35 % conversion. These drastic differences between materials – seen also in **Paper III and Paper IV** – will be further discussed below.

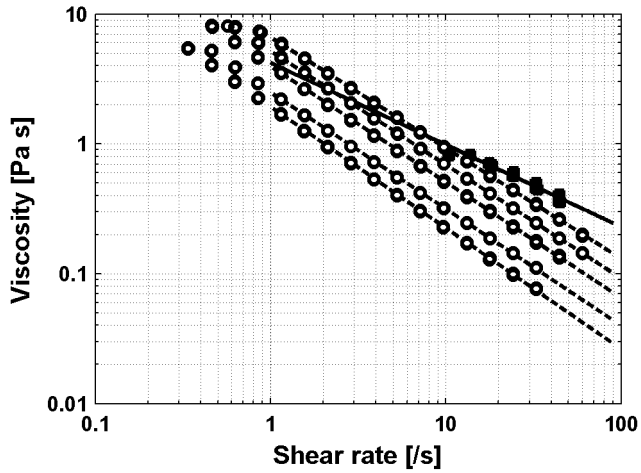

Figure 3.5. Continuous decrease of viscosity during enzymatic hydrolysis of pretreated spruce. Solid symbols represent the starting material and open symbols the decrease during hydrolysis. (Data from **Paper I**)

3.2 High solids enzymatic hydrolysis and the influence of mixing

Working at high solid loadings is generally associated with yield reductions in enzymatic hydrolysis and SSF. Numerous studies have reported these yield reductions which have been well summarized by for example Kristensen et al. (2009b) and Laveson et al. (2012). The reason for the yield decrease, which often is referred to as the "high solids effect", is not fully understood. Tentative explanations put forward have been, for example, increased end-product inhibition, enzyme adsorption, mass transfer issues and/or reduced water activity (Kristensen et al. 2009b; Roberts et al. 2011; Selig et al. 2012; Selig et al. 2013). There is most likely not a single factor which can explain this phenomenon, but rather a combination of factors. This complicates investigations since it is very difficult to isolate effects in these highly heterogeneous systems. Mixing is related to many of the suggested explanations, since in an ideally mixed system no gradients occur (either in concentrations or temperature) whereas in any non-ideally mixed system gradients will be present.

In order to effectively mix concentrated biomass slurries during hydrolysis, specially-designed reactors are needed (Andrić et al. 2010; Koppram et al. 2014). A well-studied problem which arises when mixing highly viscous fluids/suspensions in stirred tank reactors is the formation of a cavern around the impeller. This is particularly problematic if the fluids exhibit a yield stress (Wilkens et al. 2005). A cavern effectively separates the reactor volume into one well-mixed and one poorly mixed – or even completely stagnant - region where large gradients can occur. The size of the cavern is determined by the rheological properties of fluid and the design and speed of the impeller (Solomon et al. 1981; Amanullah et al. 1998).

Different reactor/mixing systems have been proposed in the literature for high solid enzymatic hydrolysis in order to circumvent poor mixing (Andrić et al. 2010; Jorgensen et al. 2007; Roche et al. 2009b; Zhang et al. 2010). A popular design is the horizontal reactor, which either rotates or contains rotating impellers (Jorgensen et al. 2007; Roche et al. 2009b). This set-up is good for handling high solid loadings, but mixing tends to get inefficient once liquefaction of the material is achieved. Additionally, horizontal reactors may have drawbacks at large scales since they typically operate at large dead-volumes, resulting in large reactor volumes and hence increased capital costs. The viability of free-fall mixing might furthermore be related to the substrate since tumble mixing has been found not to improve the performance of high solid SSF using pretreated spruce as a substrate (Hoyer et al. 2013). The use of vertical reactors will however require a well-designed impeller system in order to achieve adequate mixing (Zhang et al. 2010).

3.2.1 Reactor set-ups and mixing concepts used in this work

Two different vertical reactor systems were used within the scope of this work to study different aspects of mixing; a standard bioreactor, Biostat A+ (Braun Biotech International, Melsungen, Germany), and a specially-designed system, “Hanna” (Belach Bioteknik, Stockholm, Sweden) (Figure 3.6). The reactors are similar in size and are both equipped with central impellers. The Hanna system, however, was especially designed for high solid enzymatic hydrolysis and contains three main differences compared to the Biostat A+ system. First, the Hanna system is equipped with a stronger down-geared motor, capable of measuring torque and power input. Second, the impeller used is a wide anchor impeller (Figure 3.6 C) which almost scrapes the reactor walls. Finally, the temperature is controlled by a water jacket rather than a heating blanket. This allows a smoother temperature control to avoid large temperature gradients, which could potentially deactivate the enzymes.

Figure 3.6. Experimental set-up. The more advanced hydrolysis reactor Hanna (A) and the standard bioreactor, Biostat A+ (B). The Hanna system was equipped with an anchor impeller (C) and the Biostat reactor with a pitched blade impeller (D).

When discussing mixing a few basic concepts needs to be defined, for example stirrer torque, mixing power, impeller power number, average shear rate and Reynolds number. The power consumption, P (W), in a stirred tank reactor is a function of the dimensionless impeller power number (N_p), the fluid density, ρ (kg/m^3), the impeller speed, N (s^{-1}), and impeller diameter, D (m), according to the power equation:

$$P = N_p \cdot \rho \cdot N^3 \cdot D^5 \ (Eq. 3.2)$$

The power input can be calculated based on measured torque, T (N m), and impeller speed according to:

$$P = 2\pi \cdot T \cdot N \ (Eq. 3.3)$$

The power number, N_p, is a function of the dimensionless Reynolds number, Re, as qualitatively shown in Figure 3.7 and the Reynolds number in a stirred reactor, is a dimensionless number defined by:

$$Re = \rho \cdot N \cdot D^2/\mu \ (Eq. 3.4)$$

In the laminar region (typically Re < 10-100), Np is inversely proportional to the Reynolds number, whereas at fully turbulent conditions (Re > 10 000) Np is constant.

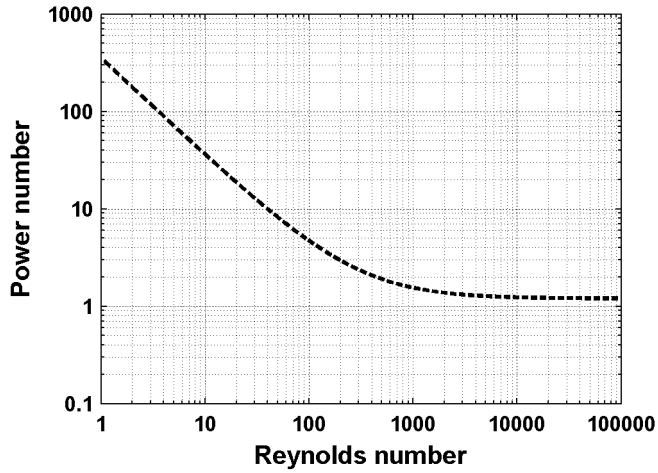

Figure 3.7. Typical representation of how the power number depends on Reynolds number.

Since the pretreated biomass slurry is known to be shear-thinning (Chapter 3.1), knowledge about the shear forces in the reactor is necessary to be able to estimate the viscosity. Metzer and Otto (1957) established an empirical relationship between impeller speed and average shear rate (γ_{avg}) in a stirred tank that is used still today:

$$\gamma_{avg} = k \cdot N \quad (Eq. 3.5)$$

The constant k is specific to the reactor and impeller design. In this work, a value of 11.5 has been used for pitched blade impellers (Wu et al. 2006) and 20 for the anchor impeller (Doran 1995). Equation 3.5 provides a simple way to estimate the average shear rate in the reactor which can then be used to calculate the average viscosity in the reactor (if the power law parameters for the material are known) and the average Reynolds number.

3.2.2 Influence of mixing on enzymatic hydrolysis of pretreated spruce

A central result in **Paper II-V** is the strong positive effect of mixing for high solids enzymatic hydrolysis of steam pretreated spruce. This effect was first reported in **Paper II**, where a comprehensive quantitative study of the influence of agitation rate on the hydrolysis of steam pretreated spruce was performed. It was shown that the hydrolysis rate could easily be affected as much by stirring as by doubling the enzyme loading (Figure 3.8). For example, the conversion after 96 hours of hydrolysis was twice as high at 500 rpm as compared to that at 75 rpm. Similar observations, although not as strong, have been reported previously for pretreated spruce (Hoyer et al. 2009; Mais et al. 2002; Tengborg et al. 2001). For other materials, contradicting findings on the effect of mixing have been reported (Jorgensen et al. 2007; Roche et al. 2009b). In any case, an efficient initial distribution of the enzymes is usually regarded as crucial (Roche et al. 2009b).

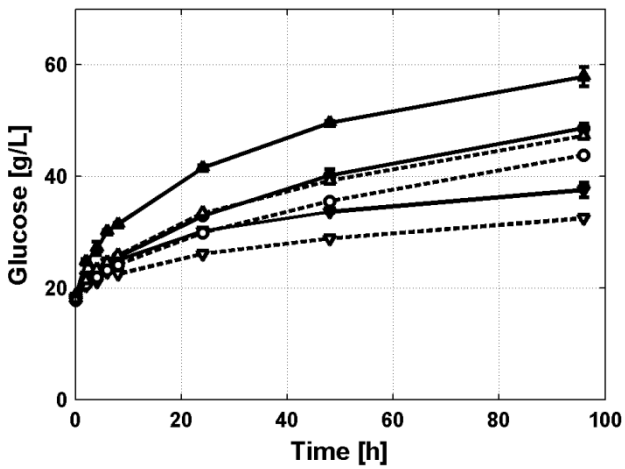

Figure 3.8. Enzymatic hydrolysis of pretreated spruce at 500 rpm (▲), 300 rpm (●) and 75 rpm (▼). Solid symbols/lines represents an enzyme load of 20 FPU/g glucan and open symbols/dotted lines represents a load of 10 FPU/g glucan. (Data from **Paper II**)

An increased agitation rate induces many changes in the reactor. Since the material typically is shear-thinning, the viscosity falls due to increased shearing, and the Reynolds number increases. At the same time, power consumption increases. The objective of **Paper III-IV** was to gain a better understanding of the underlying phenomena by measuring the actual mixing power input and PSD during the hydrolysis for different lignocellulosic materials.

3.2.3 Torque and mixing power during enzymatic hydrolysis

The Hanna reactor (section 3.2.1) enabled the effect of mixing on higher WIS loadings to be studied. As previously mentioned, this system is also capable of measuring torque on the stirrer shaft, which provides valuable information about needed mixing power and viscosity changes during hydrolysis (**Paper III**). By comparing the behavior of two rather different raw materials, i.e. spruce and giant reed, during the course of hydrolysis it became obvious that different biomass respond rheologically quite different to enzymatic hydrolysis, and the rate of viscosity reduction is highly material specific (Figure 3.9A-B). Moreover, the drop in viscosity was not necessarily coupled to the glucose yield in a simple manner, especially for the pretreated giant reed (Figure 3.9).

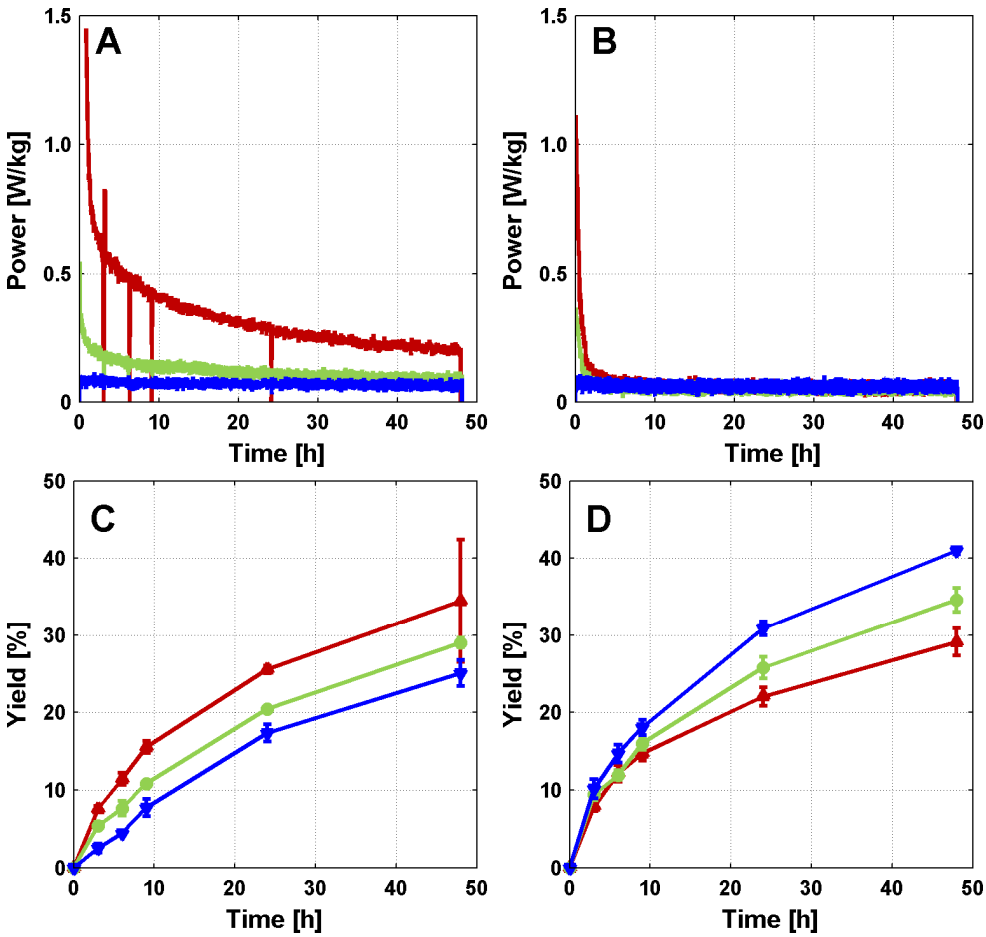

Figure 3.9. Power consumptions and glucose yields during enzymatic hydrolysis of spruce (left) and giant reed (right). Red represents 20 % WIS, green 15 % WIS and blue 10 % WIS. (Data from **Paper III**)

Similar results have recently been reported for wheat straw, where the viscosity reduction could be correlated to the hydrolytic action of endoglucanase rather than complete hydrolysis to glucose (Skovgaard et al. 2014). This is also in accordance with the quick drop in yield stress found by Roche et al. (2009a) for pretreated corn stover. Spruce however retains its high viscosity for a longer time, despite glucose yields in the same range as in the case of giant reed (Figure 3.9C-D).

Surprisingly, a faster enzymatic conversion was achieved at higher initial WIS content for spruce (Figure 3.9C), contrary to common knowledge. This could, however, be related to the much higher power input needed to operate the impeller. Controlling the mixing by applying a controlled specific power input (instead of stirrer speed), regardless of WIS loading, resulted in a shift of the hydrolysis trend and the expected lower conversion was found with increasing WIS loadings (Figure 3.10). Changing the mixing power (and hence stirrer speed), however, had no significant impact on the hydrolysis of giant reed, indicating yet another fundamental difference between the two materials. A lack of enhanced hydrolysis performance at improved mixing conditions has previously been shown for both corn stover (Roche et al. 2009b; Samaniuk et al. 2011) and wheat straw (Jorgensen et al. 2007), two materials with more similarities to giant reed than spruce.

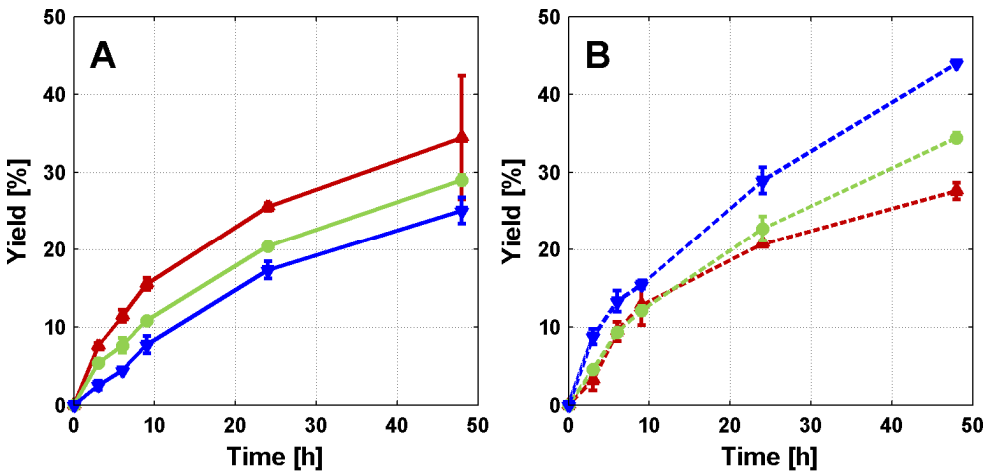

Figure 3.10. Difference in running the hydrolysis of spruce at the same rpm (A) or same power input (B) for different WIS loadings. Red – 20 % WIS, green – 15 % WIS and blue – 10 % WIS. (Data from **Paper III**)

3.2.4 Agitation effects on particle size distribution (PSD)

Another difference between giant reed and spruce was observed in **Paper IV**, where PSD changes were followed during the course of hydrolysis. Giant reed consisted of much larger particles than spruce, but more interestingly, the mean particle size decreased rapidly during hydrolysis. Moreover, this decrease was rather independent of impeller speed (Figure 3.11). The rapid reduction in particle size, likely explains the quick liquefaction observed for giant reed, since reduction in viscosity and yield stress has previously been correlated to smaller particle sizes for similar materials, i.e. corn stover (Ehrhardt et al. 2010; Viamajala et al. 2009) and wheat straw (Skovgaard et al. 2014).

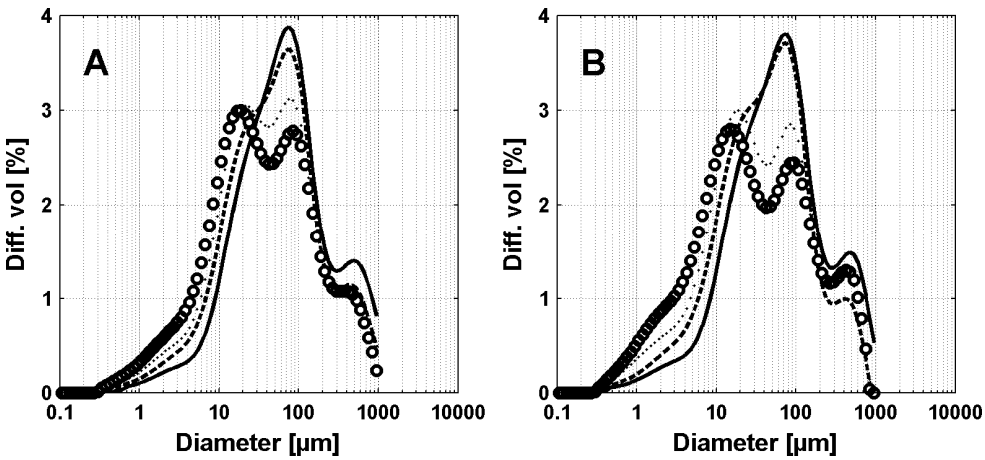

Figure 3.11. PSD changes during hydrolysis of giant reed at 100 rpm (A) and 300 rpm (B). Solid line – 0h, dashed line – 4h, dotted line – 24h, circles – 96h. (Data from **Paper IV**)

During spruce hydrolysis the change in PSD was much more dependent on impeller speed and only a minor decrease occurred at low impeller speeds (Figure 3.12A-C). The changes in PSD were actually not correlated to the hydrolysis but rather to the mechanical action of the impeller at high speeds (Figure 3.12D). Presumably, the shear forces in the reactor where strong enough to induce a mechanical break-up of the fiber complex, effectively reducing the mean particle size and changing the PSD. The change in PSD at high agitation rates was also found when the WIS content was lowered from 13 % to 7 %. Interestingly though, the effect of mixing on the hydrolysis rate diminished once the WIS content was reduced (Figure 3.13).

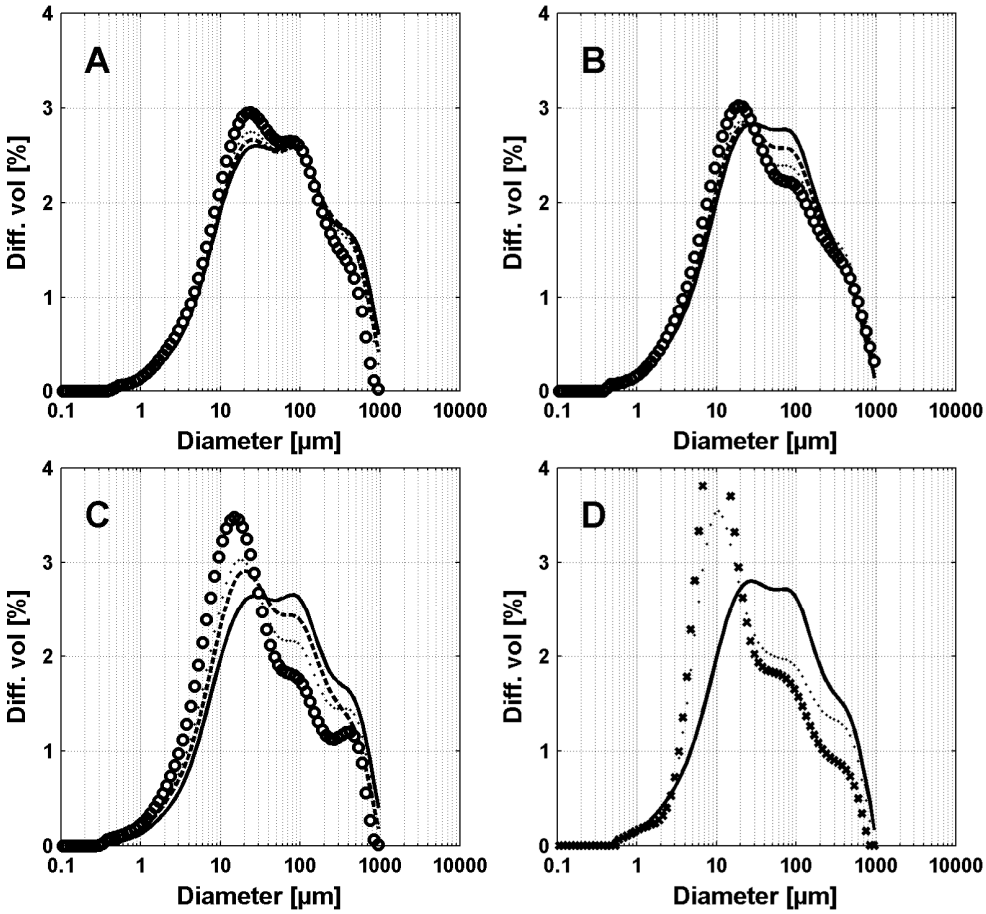

Figure 3.12. PSD changes during hydrolysis of spruce at 100 rpm (A), 300 rpm (B), 600 rpm (C) and during pure agitation without added enzymes (D). Solid line – 0h, dashed line – 4h, dotted line – 24h, cross – 48h, circles – 96h. (Data from **Paper IV**)

A small initial increase in hydrolysis rate, at the higher agitation rate, was also observed for the hydrolysis of giant reed at 13 % WIS loading (Figure 3.13), which in a way contradicts the findings of **Paper III**. The discrepancy can likely be explained by the use of a different and less efficient mixing system in **Paper IV** compared to **Paper III**, also indicated by a longer liquefaction time (up to 24 hours). Once liquefaction was achieved, no significant difference could be found between the different stirrer speeds for giant reed hydrolysis.

The combined results of **Paper III** and **Paper IV**, i.e. that the effect of agitation on the enzymatic hydrolysis of pretreated spruce diminishes when lowering the solids loading, and that the effect is rather absent for the rapidly liquefied giant reed, point towards the assumption that it is the viscosity of the material that determines whether or not mixing affects the hydrolysis rate.

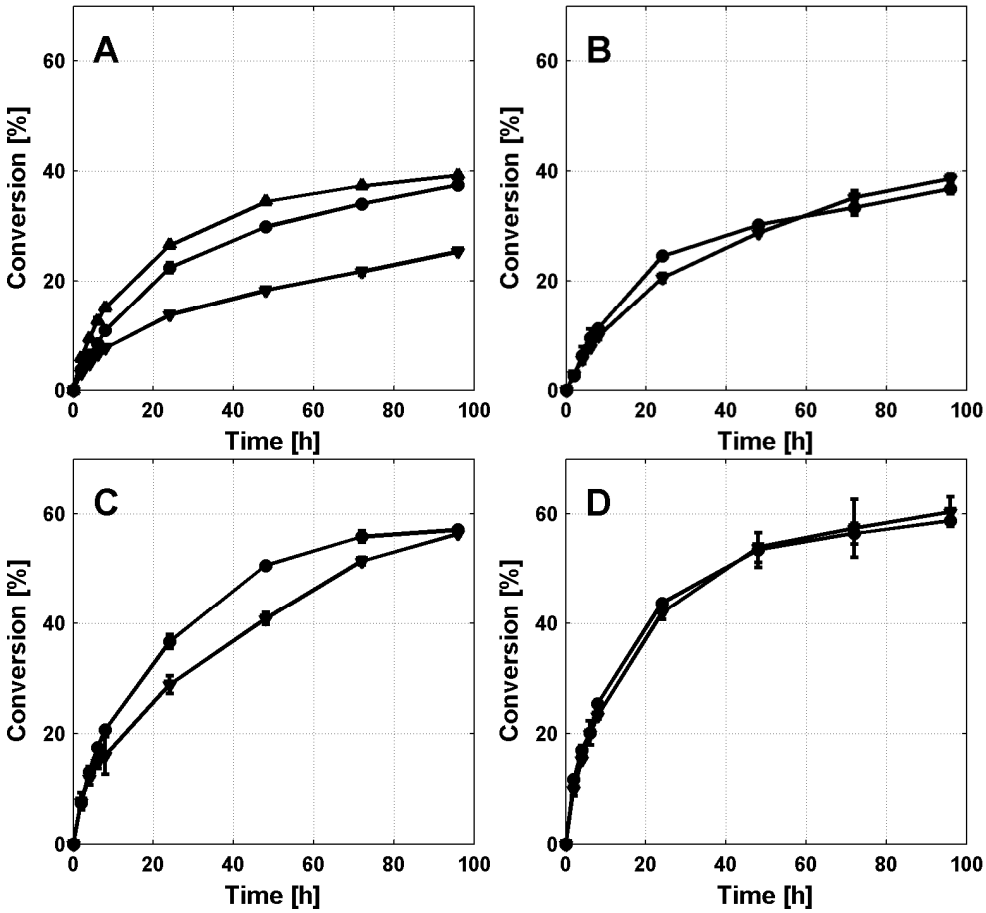

Figure 3.13. Hydrolysis yield at 13 % WIS (A, C) and 7% WIS (B, D) for spruce (A, B) and giant reed (C, D). ▲ = 600 rpm, ● = 300 rpm, ▼ = 100 rpm. (Data from **Paper IV**)

3.3 Scaling up enzymatic hydrolysis

When increasing the production scale, a number of different process parameters will change, and several of these could affect the hydrolysis process, such as flow regime and specific power input. The flow regime is strongly dependent on the viscosity of the material and will also depend on the scale, according to equation 3.4. In **Paper V**, spruce hydrolysis was assessed and compared at different scales, allowing the effect of specific power input and Reynolds number to be (partly) separated.

The effect of mixing on the enzymatic hydrolysis of pretreated spruce was found to remain during scale up, at least up to cubic meter scale, as confirmed for two different pretreatment batches (Figure 3.14).

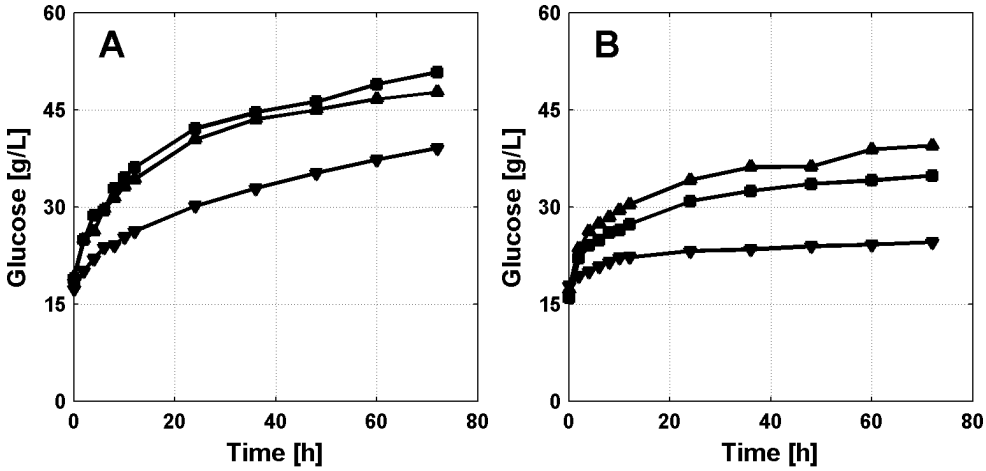

Figure 3.14. Enzymatic hydrolysis at demonstration scale for two pretreated spruce materials at 120 rpm (▲), 60 rpm (■) and 30 rpm (▼). (Data from **Paper V**)

Mixing power consumptions were monitored during the experiments and scale-down experiments, based on specific power inputs, were then performed in the Hanna system using different sets of impellers (pitched blade and anchor). The hydrolysis rates were substantially higher at the larger scale at similar power input (Figure 3.15). These results are encouraging for large scale implementation, since prohibitively high (economically-speaking) mixing power consumptions have been reported in lab scale by, for example, Zhang et al. (2010). The better performance found here at large scale is thus highly significant.

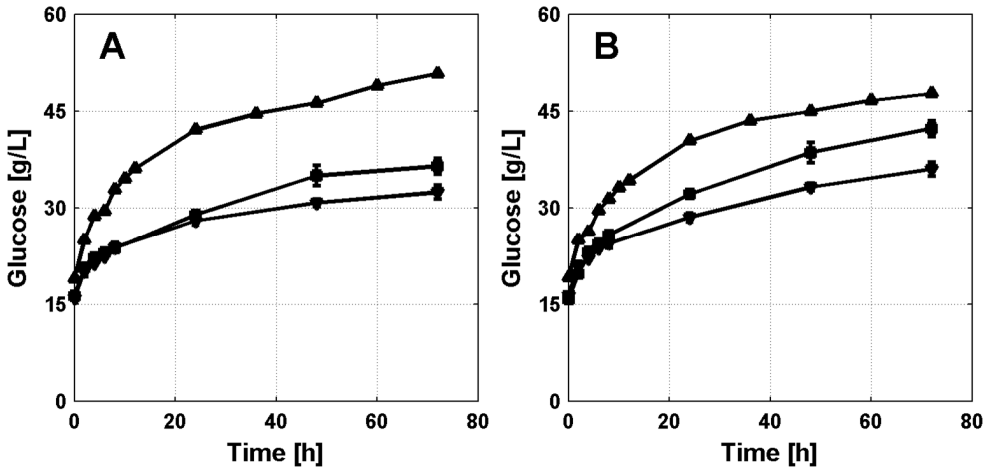

Figure 3.15. Influence of scale and impeller configuration for enzymatic hydrolysis at the specific power input of 0.23 kW/m^3 (A) and 0.45 kW/m^3 (B) at demo scale using pitched blade impellers (▲), at lab scale using anchor impeller (■) and at lab scale using pitched blade impellers (▼). (Data from **Paper V**)

Furthermore, it was found that when operated at the same specific power input the choice of impeller affected the hydrolysis rate (Figure 3.15), *which indicates the importance of the flow pattern*. As discussed briefly earlier, an anchor-shaped impeller provides a completely different flow pattern in the reactor compared to pitch blade impellers, and the former seems favorable for the enzymatic hydrolysis – at the same specific power input. Since the viscosity was measured for the pretreated material, estimations of average Reynolds number could be done for all experiments by estimating the shear rate in the reactor, according to equation 3.5. Throughout all the experiments, regardless of scale and mixing equipment, hydrolysis yields were better correlated with Reynolds number than specific power input (Figure 3.16). Again, this highlights the importance of flow region for the enzymatic hydrolysis of pretreated spruce.

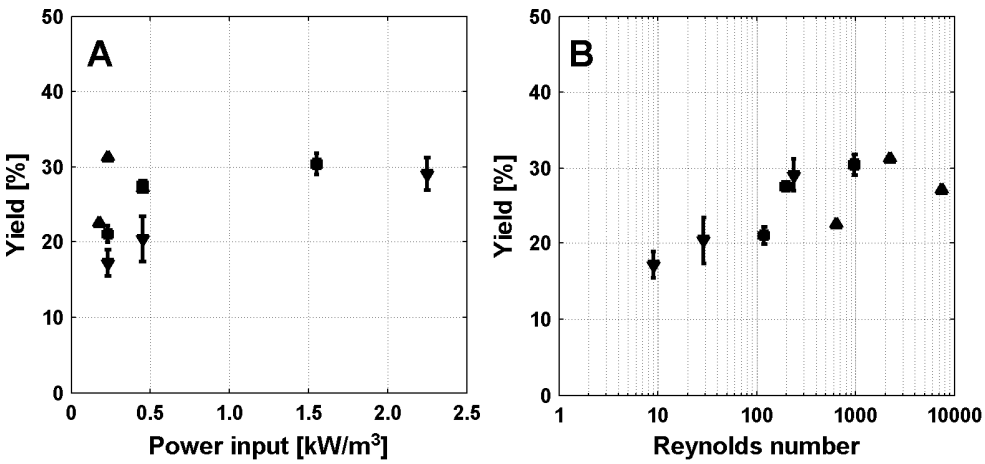

Figure 3.16. Enzymatic hydrolysis yield vs. power input (A) and Reynolds number (B) for different scales and impeller configurations, i.e. demo scale using pitched blade impellers (▲), lab scale using anchor impeller (■) and lab scale using pitched blade impellers (▼). (Data from **Paper V**)

3.4 Final remarks and thoughts

The work carried out in **Paper I-V** clearly highlights the large differences in terms of rheology and flow behavior between different materials, and the importance of rheology for designing enzymatic hydrolysis. Large rheological differences were also found within differently pretreated spruce materials. Many of these phenomena seem correlated to PSD and especially changes in PSD during hydrolysis. Furthermore, the rheological discrepancy between materials likely explains the different responses to mixing during enzymatic hydrolysis.

The scale-up study in **Paper V** nicely illustrates the need to study mixing aspects in multiple set-ups. Although the mechanistic reason behind the different impacts

of mixing on enzymatic hydrolysis, is not yet fully understood, the results of this work indicate that mass transfer effects – caused by flow conditions at high viscosities – limit the enzymatic hydrolysis. Increased mass transfer rates would increase the transport of produced sugars, mainly cellobiose, from the fibers out to the bulk liquid, hence reducing local end-product inhibition. Also, enzyme transport could possibly be enhanced during their repeated desorption/re-adsorption action. These phenomena would likely be more important during enzymatic hydrolysis of spruce, in comparison to grass materials, since spruce retains its high viscosity for long times during enzymatic hydrolysis

Chapter 4

Designing high solids bioethanol processes for enhanced xylose utilization

In the previous chapter, the challenges of high solid processing and enzymatic hydrolysis were discussed, focusing mainly on the importance of rheological properties and mixing. In addition to increased viscosities and mixing problems, high solid operations also result in increased concentrations of biomass degradation products, such the furaldehydes (HMF and furfural) and acetic acid. These compounds can potentially inhibit the fermenting micro-organism (Almeida et al. 2011). Furthermore, when working with xylose rich materials, xylose consumption will be affected by both higher glucose concentrations and increased concentrations of inhibitors.

In this chapter, which is based on **Paper VI-VII**, different process designs to handle xylose rich materials, specifically wheat straw and giant reed, are discussed. Emphasis is placed on enhancing xylose conversion while at the same time working at high solid loadings.

4.1 General process designs

Much progress has been achieved to successfully introduce xylose metabolism in *S. cerevisiae* (as discussed in chapter 2.2.3) and several commercial actors now offer industrial strains with xylose fermenting abilities (e.g. Butalco[7], C5LT[8], DSM[9], Taurus Energy[10], Terranol[11]). However, glucose is still the preferred substrate, and xylose consumption in *S. cerevisiae* is hampered by high glucose concentrations (Lee et al. 2002; Saloheimo et al. 2007). It has, however, also been shown that a low co-consumption of glucose is beneficial for xylose consumption (Meinander et al. 1999), which may be coupled to the uptake kinetics and expression of transporters (Bertilsson et al. 2008). Certainly, the issue of improved xylose uptake continues to be addressed by genetic engineering by, for example, the introduction of new sugar transporters (Fonseca et al. 2011; Runquist et al. 2009; Runquist et al. 2010). However, it is also quite clear that process design will influence the xylose (co-)consumption.

When comparing the main process options, SSF or SHF (see Chapter 2.2.4), an SSF (or SSCF where "C" indicates co-fermentation of xylose) concept will likely be favored over SHF (or SHCF). The reason is that glucose concentrations are kept lower, while at the same time glucose is continuously released to the liquid phase through the enzymatic hydrolysis, thereby enabling co-consumption of glucose and xylose (Olofsson et al. 2008a).

With these traits in mind, intelligent process designs have been proven very useful for enhancing xylose consumption during co-consumption with glucose. Some of the different successful strategies that have been demonstrated includes; i) pre-fermentation as a means to reduce unfavorable high glucose concentrations (Bertilsson et al. 2009), ii) enzyme feeding to control glucose release rates (Olofsson et al. 2010), iii) feeding of hydrolysate liquid to maintain low and steady glucose concentrations (Erdei et al. 2012, 2013) and iv) substrate feeding as a way to allow a higher total WIS loading by avoiding mixing problems and enable in-situ detoxification (Olofsson et al. 2008b). The various approaches can be summarized as shown in (Figure 4.1).

[7] http://www.butalco.com/ (2014-03-27)

[8] http://c5lt.se/ (2014-03-27)

[9] http://www.dsm.com/ (2014-03-27)

[10] http://www.taurusenergy.eu/ (2014-03-27)

[11] http://www.terranol.com/ (2014-03-27)

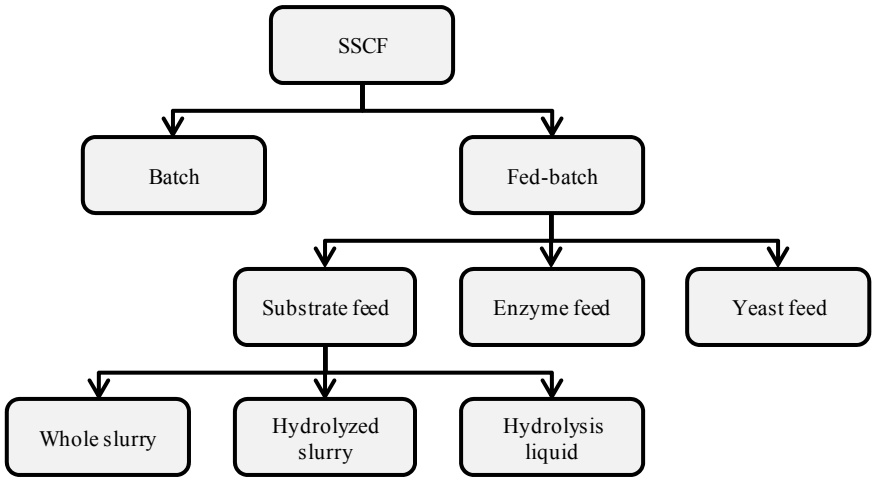

Figure 4.1. Schematic of different potential feeding methods.

Instead of employing a fed-batch process to handle high solid loadings, another option is to include a high temperature hydrolysis step, prior to an SSCF, which takes place at lower temperature. This is often referred to as *viscosity reduction* (Jorgensen et al. 2007). In this way, part of the enzymatic hydrolysis can be optimized separately from the fermentation process. If the length of the high temperature hydrolysis step is prolonged, it can be referred to as a *pre-hydrolysis* step. If it is extended even further, to almost complete hydrolysis, the process becomes an SHCF process, with the exception being that the solids remaining after hydrolysis are not removed. This concept, of a high temperature partial hydrolysis, will in this dissertation be referred to as a *hybrid process* (see Figure. 4.2). With new improved enzyme blends, this hybrid approach has been reported as beneficial in comparison to SSF for hexose fermentation in high solids ethanol production from wheat straw (Cannella and Jørgensen 2014). The increased temperature stability and decreased end-product inhibition of the enzymes speak in favor of the hybrid process.

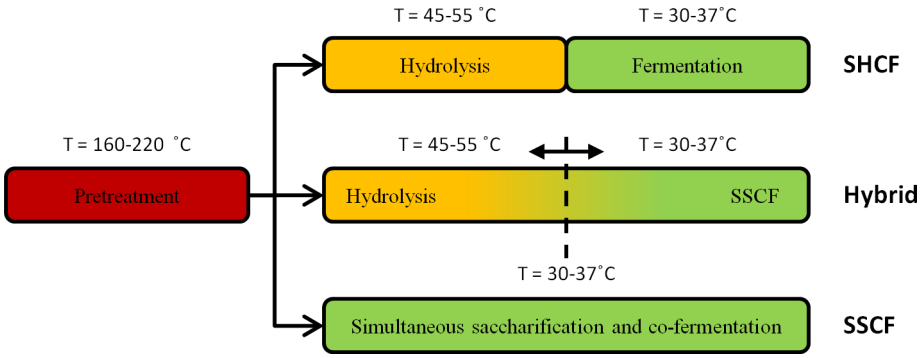

Figure 4.2. Illustration of the hybrid process concept.

4.2 Designing dual feeding strategies for enhanced xylose conversion

In **Paper VI**, different combinations of substrate and enzyme feeds were investigated as means to increase the xylose consumption and final ethanol titer using pretreated wheat straw. As a reference case pure substrate feeding was used, since unfortunately no batch reference could be made due to severe mixing problems at the WIS content used. Substrate feeding has, however, previously been shown to be superior to batch fermentations for similar set-ups, especially at elevated WIS content (Olofsson et al. 2008b).

In **Paper VI,** a final WIS loading of 11 wt-% was achieved by substrate feeding (starting at 8 %) using standard fermentors (Biostat A+). Since rather high glucose concentrations were observed for the reference case, the substrate feed was combined with enzyme feeding using three different feed profiles (A, B, C) (Figure 4.3). The intention was to better control the release rate of glucose and to maintain glucose concentration closer to zero, thus promoting xylose consumption.

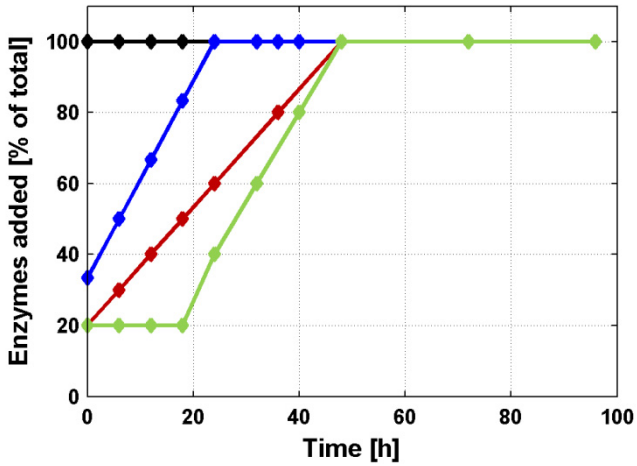

Figure 4.3. The enzyme feed profiles during SSCF for the references case (black) and feed case A (blue), B (red) and C (green). (Data from **Paper VI**)

With combined feeding of enzymes and substrate, an increase in xylose consumption from 40 to 50 % could be achieved for the best case (B). This resulted in a more than 10 % increase in final ethanol concentration (from 33 to 37.5 g L^{-1}), Figure 4.4. However, this type of feeding requires careful thought, as only one of the proposed feeding schemes (B) actually improved the final ethanol titer (Figure 4.4). For case A, the enzyme feeding was probably too fast, since a similar glucose profile was observed as for the reference case. Consequently, this resulted in both similar xylose uptake and final ethanol concentration. Contrary, in case C, the enzyme feeding was likely too slow. Even though the low glucose concentrations enhanced *xylose uptake* compared to the reference, the initial ethanol production was significantly lower due to a reduced hydrolysis rate. The hydrolysis was never able to 'catch up' since the enzymes did not have enough time for carrying out the hydrolysis (in comparison to the reference and feeding profile B). A similar strategy with combined substrate and enzyme feed, including a pre-fermentation step, was recently successfully implemented at demonstration scale using steam pretreated corn cobs (Koppram et al. 2013).

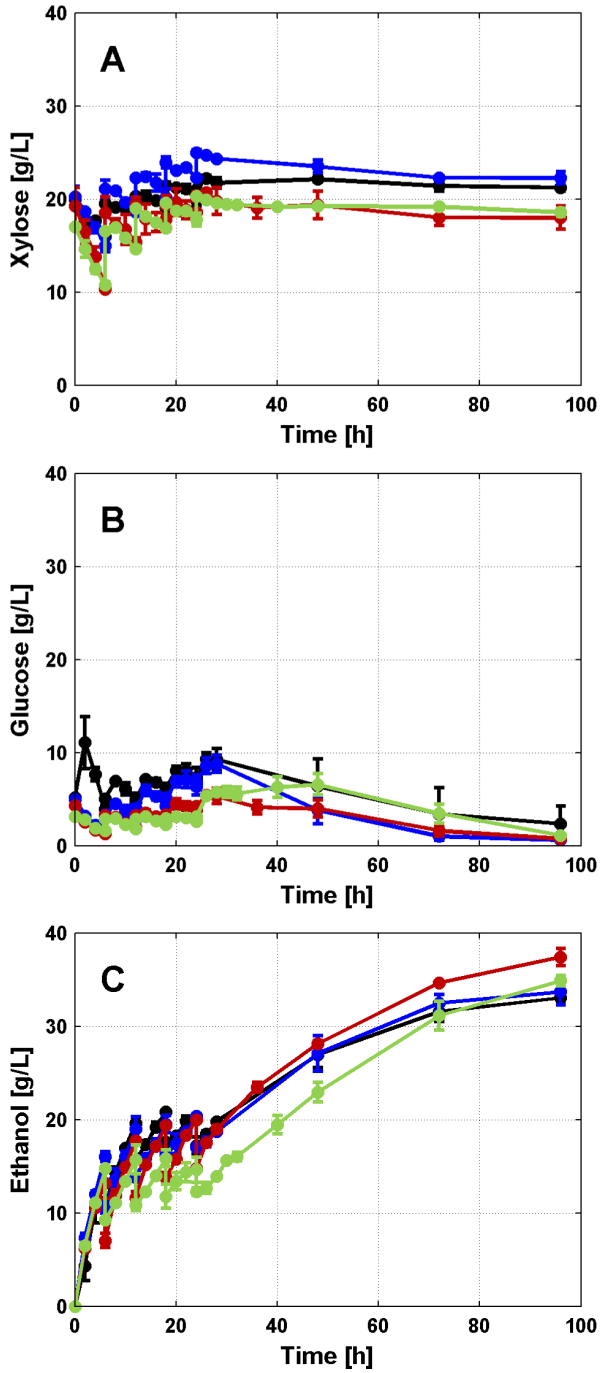

Figure 4.4. Concentration profiles during SSCF for the references case (black) and the dual feed case A (blue), B (red) and C (green). (Data from **Paper VI**)

4.3 Hybrid process designs

In **Paper VII**, a hybrid process design was evaluated and compared to standard SSCF for the conversion of giant reed to ethanol. As previously mentioned, hybrid processes, with a high temperature hydrolysis step, are beneficial for the enzymatic hydrolysis – unless strongly inhibited by either high sugar concentrations or other compounds which undergo microbial conversion in the fermentation. However, as also discussed above, the ratio between glucose and xylose concentrations will be higher in such a process, thereby affecting xylose uptake. The hybrid process design evaluated in **Paper VII** consisted of a 48-hour long hydrolysis phase at 45 ˚C, followed by a 48-hour long SSCF phase at 34 ˚C. Pure SSCF experiments were run for 96 hours (at 34 ˚C), hence keeping the same total reaction time.

4.3.1 Temperature and hydrolysis in a hybrid process

The main compromise in an SSCF process is that the temperature has to be kept at a sub-optimal level for the enzymes. This is illustrated in Figure 4.5, where hydrolysis data for pretreated giant reed at 45 ˚C (for 48 h) is shown together with hydrolysis data at 34 ˚C (for 96 h) (from **Paper VII**). There is obviously a strong positive effect by temperature on the rate of enzymatic hydrolysis.

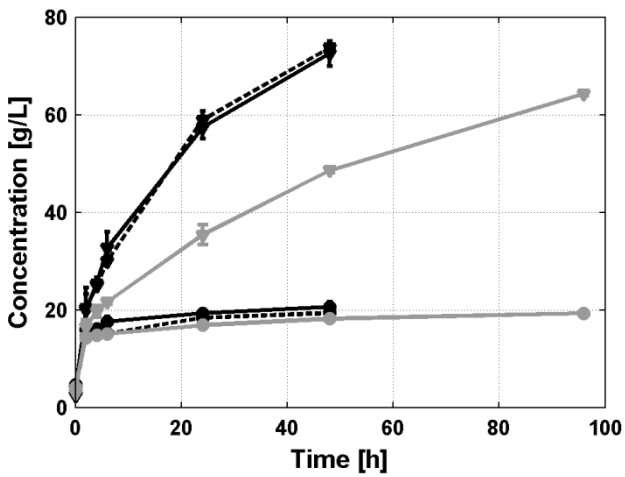

Figure 4.5. Concentration profiles for glucose (▼) and xylose (●) during hydrolysis of giant reed at 45 ˚C (black) and 34 ˚C (grey). (Data from **Paper VII**)

It is worth pointing out that the glucan conversion (determined by post-fermentation material analysis) in the SSCF process was significantly higher than that for the pure hydrolysis *at the same temperature* (Table 4.1). However, the conversion was still highest, when a high temperature hydrolysis step was used. Nonetheless, the results show that SSF, even with the improved enzymes of today, still reduces end-product inhibition. This speaks in favor of the hybrid concept over pure SHF (where the solid fraction is removed) since it is likely that the hydrolysis can be pushed a little further once the sugars have been fermented.

Table 4.1. Glucan conversion determined after 96 hours for different process configurations and temperatures. (Data from **Paper VII**)

	Hydrolysis	**SSCF**	**Hybrid**
Temperature	34 ˚C	34 ˚C	45 ˚C (0-48 hour), 34 ˚C (48-96 hour, SSCF)
Glucan conversion (%)	45.2±0.3	49.4±0.8	54.2±0.5

4.3.2 Fermentation at different pH in a hybrid process

The (glucan) hydrolysis is not the only concern, as the fermentation of all sugars is critical for the process. For the fermentation, the ratios of sugars, as well as inhibitors, affect the fermentation (as discussed in chapter 2.2.3).

In the case of giant reed, the feedstock in **Paper VII**, acetic acid was identified as the most prominent inhibitor with the pretreatment used. As discussed in chapter 2.2.3 acetic acid is a strong inhibitor, in particular for xylose metabolism, but also for glucose metabolism. The effect of acetic acid is strongly coupled to pH since it is the un-dissociated form of the acid that diffuses across the cell membrane and dissociates inside the cell due to the higher intracellular pH (Casal et al. 1996). The cost of maintaining the intracellular pH is ATP, to fuel the hydrogen pumps to transport hydrogen ions out of the cell. Low levels of acetic acid are not necessarily bad for the process though, since it has been shown that fermentation rates *as well as* product distribution in the cell are affected by the level of acetic acid inhibition. At zero or very low levels of acetic acid, cell growth and glycerol production is typically favored which results in lower ethanol yields (Taherzadeh et al. 1997). Slight increases in acetic acid levels thus results in increased ethanol yields. Low amounts of acetic acid, for example, has been found to be beneficial when combining 1st and 2nd generation ethanol production from wheat meal and straw (Erdei et al. 2010). When strong inhibition occurs though, fermentation rates are severely hampered.

The sensitivity of acetic acid inhibition to pH was confirmed for TMB3400 in hydrolysis liquid supplemented with acetic acid (Table 4.2), where relatively small changes in pH clearly affected fermentation. At the most severe conditions, an acetic acid level of 8 g/L and a pH of 5.0 (the lowest pH tested), the fermentation

rate of glucose was severely affected and only a minor xylose uptake was found (Figure 4.6). With 4 g/L acetic acid, no significant difference in glucose uptake rate was found, although xylose consumption was strongly affected by the pH (Table 4.2).

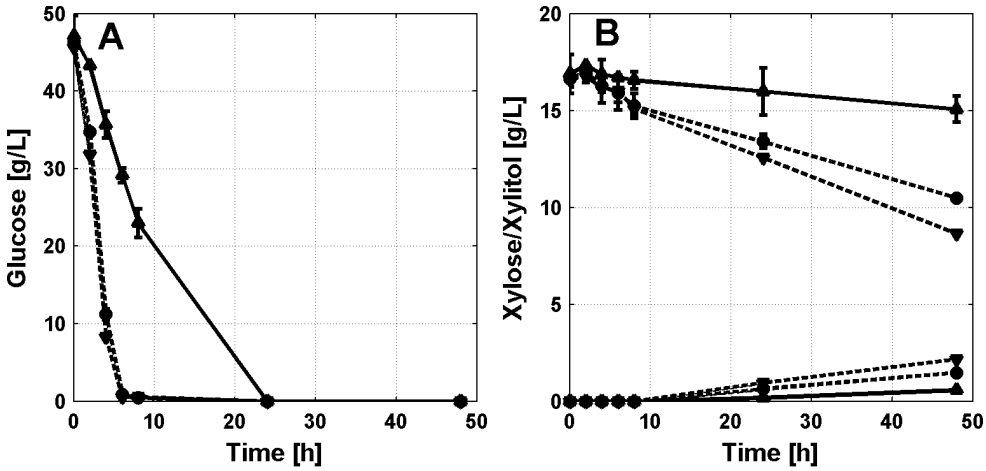

Figure 4.6. Influence of pH on glucose (left) and xylose (right) uptake during shake flask fermentations of hydrolysis liquid with 8 g/L acetic acid, at pH 5.0 (▲), pH 5.5 (●) and pH 6.0 (▼). (Data from **Paper VII**)

Table 4.2. Fermentation performance during shake flask fermentations of hydrolysate liquid at different pH and acetic acid concentrations. Values labeled with the same letter are not significantly different at a confidence level of 95%. Note that in this table, yields for all six set-ups are compared. (Data from **Paper VII**)

	4 g/L acetic acid		
	pH 5.0	pH 5.5	pH 6.0
Ethanol yield (g/g consumed sugars)	0.44 ±0.01^{A}	0.43 ±0.00^{AB}	0.40 ±0.02^{C}
Ethanol yield (% of theoretical)	85.5 ±1.2	83.8 ±0.6	78.5 ±3.6
Glycerol yield (g/g consumed sugars)	0.043±0.007	0.042±0.002	0.045±0.004
Consumed xylose (%)	27.4 ±1.9^{C}	38.7 ±2.5^{A}	50.2 ±1.2^{B}
Xylitol production (% of consumed xylose)	23.9 ±0.2	25.2 ±4.0	28.5 ±2.6
Calculated carbon recovery[1]	0.99 ±0.00	0.99 ±0.01	0.97 ±0.04
	8 g/L acetic acid		
	pH 5.0	pH 5.5	pH 6.0
Ethanol yield (g/g consumed sugars)	0.43 ±0.03^{AD}	0.43 ±0.01^{AD}	0.42 ±0.01 ^{BCD}
Ethanol yield (% of theoretical)	84.9 ±5.4	84.5 ±1.9	82.3 ±0.9
Glycerol yield (g/g consumed sugars)	0.039±0.009	0.042±0.003	0.046±0.000
Consumed xylose (%)	10.7 ±1.2^{D}	37.0 ±0.6^{A}	48.0 ±2.0^{B}
Xylitol production (% of consumed xylose)	32.1 ±7.8	23.8 ±0.7	27.2 ±2.0
Calculated carbon recovery[1]	0.95 ±0.04	0.99 ±0.01	1.01 ±0.02

[1] For details about carbon recovery, refer to **Paper VII**.

Since this strong effect of pH on the fermentation was found, both the hybrid process design and the SSCF process were evaluated at different fermentation pH. The beneficial effect of increasing the pH was found to be rather different depending on process design. During both designs an increased xylose uptake was observed at a higher pH (Figure 4.7), however, the increase was significantly larger for the SSCF case (Table 4.3). This was most likely due to the much (2x) longer fermentation time and the fact that low glucose levels were achieved throughout the fermentation in the SSCF.

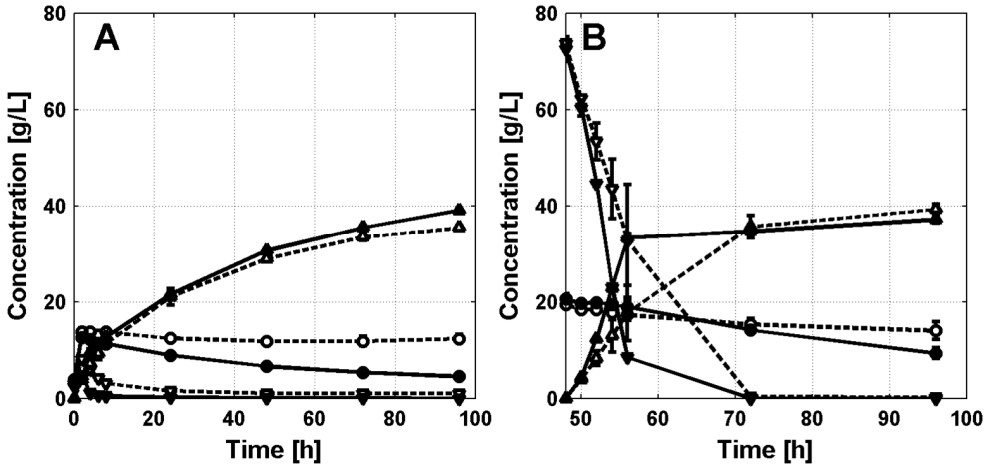

Figure 4.7. Fermentation performance during SSCF (A) and the hybrid design (B) at a pH of 5.0 (open symbols/dotted line) and 5.5 (solid symbols/lines). ▲ = ethanol, ● = xylose, ▼ = glucose. (Data from **Paper VII**)

The overall result of running the hybrid process design at the higher pH was actually a similar (or even slightly lowered) final ethanol titer, despite the increased xylose uptake. This indicates a lower fermentation yield (similar to the shake flask fermentations), since post-fermentation material analysis showed similar glucan conversions (Table 4.3). However, for the SSCF design the higher pH resulted in a 10 % increase in ethanol titer (from 35 to 39 g L^{-1}). This was likely due to the better improvement in xylose uptake, compared to the hybrid design. An indication of a slightly less affected fermentation yield at increased pH was also found for the SSCF design (although not statistically confirmed). This may be due to the fermentation rate in this design being limited by the hydrolysis rate, meaning that the cells might not be able to take full advantage of the less inhibiting environment provided by the higher pH.

Table 4.3. Hydrolysis and fermentation performance for the different process designs evaluated in **Paper VII**. Values labeled with the same letter are not significantly different at a confidence level of 95%.

	SSCF pH 5.0	**SSCF pH 5.5**	**Hybrid pH 5.0**	**Hybrid pH 5.5**
Glucan conversion (%)	49.4±0.8	50.9±3.8	54.2±0.5	54.0±1.1
Ethanol yield (g/g consumed sugars)	0.42±0.01^{A}	0.40±0.02^{A}	0.43±0.00^{A}	0.39±0.02^{A}
Ethanol yield (% of theoretical)	81.7±1.9	79.3±4.7	85.0±0.1	76.4±3.5
Consumed xylose (%)	40.2±3.8	78.3±2.0	33.7±9.2	55.8±5.5
Xylitol production (% of xylose consumed)	32.0±0.1	25.5±0.2	15.9±0.2	19.2±0.4
Overall ethanol yield (% of theoretical)	40.3±0.7^{A}	44.4±0.4^{B}	45.2±1.3^{B}	42.7±0.8^{AB}
Calculated carbon recovery[1] (excluding cell growth)	0.93±0.01	0.92±0.03	0.94±0.00	0.90±0.02

[1] For details about carbon recovery, refer to **Paper VII**.

4.4 Evaluating experiments – Calculating yields and mass balances

Calculation of yields and mass balances are always of fundamental importance for an engineer. When working in high WIS systems these calculations are not inconsequential. Most often yields are calculated based on measured sugar concentrations, typically by high-performance liquid chromatography (HPLC), taken from the supernatant of the slurry. It is normally assumed (and more or less articulated in the research available) that the density of the liquid (ρ_{liq}) is constant at 1000 g/L (water) throughout the reaction, and furthermore that the volume does not change. This result in the following equation:

$$Y_{glucan} = \frac{[Glu] + 1.0526 \cdot [Cel]}{1.111 \cdot m_{reac} \cdot WIS_0 \cdot x_{glucan_0}} \quad (Eq. 4.1)$$

m_{reac} is the total reaction mass, WIS_0 is the fraction of insoluble solids at start and xglucan is the fraction of glucan in the WIS (note: for the sake of simplicity, the examples in this section apply to washed fibers and do not take into account oligomers larger than cellobiose).

Good yield estimations are obtained with this simple equation for low WIS concentrations (< 5 % WIS), using glucose and cellobiose concentrations (g/L) given directly by HPLC. However, for higher WIS contents the accuracy is insufficient and large (up to more than 30 %) overestimations of the yield will be introduced (Hodge et al. 2009; Kristensen et al. 2009a; Roche et al. 2009b; Zhang and Bao 2012; Zhu et al. 2011). The critical assumption, responsible for the major part of the error, is however *not* the assumption of constant density and volume throughout the reaction, but rather the assumption that 1.00 kg of total reaction mass equals a liquid volume of 1.00 L. From a simple mass balance, the liquid volume should rather be calculated as the total mass minus the mass of WIS, divided by the density of the liquid phase. This results in the correct yield calculation given in equation 4.2.

$$Y_{glucan} = \frac{([Glu] + 1.0526 \cdot [Cel])}{1.111 \cdot WIS_0 \cdot x_{glucan_0}} \cdot \frac{1 - WIS}{\rho_{liq}} \quad (Eq. 4.2)$$

To calculate the true glucan conversion, one would thus have to measure both the WIS content and density of the liquid phase after the experiment. To ease the experimental procedure, one could assume a constant density and use the starting WIS concentration instead of the final one. This significantly reduces the error compared to using equation 1, but, more importantly, results in a slightly underestimated yield, rather than a large overestimation. Equation 4.2 can be used analogously for ethanol yield calculations. Moreover, the same approach should be used when calculating mass balances over the given process step.

4.5 Final remarks and thoughts

When it comes to process design, every process is unique and has to be optimized individually based on what type of raw material, yeast and enzymes are used – making it difficult to draw any general conclusions about one strategy being better than the other. However, when considering co-fermentation of xylose, the SSCF approach in general seems to give additional benefits.

It is evident though that process optimization can enhance xylose co-consumption significantly, with increased ethanol titers as a result. However, this is not an easy task, especially since a balance is needed between xylose uptake and hydrolysis yield. An interesting idea would be to utilize two different enzyme preparations. One preparation of endoglucanases to be used in a *viscosity reduction* step to

effectively reduce the viscosity of the biomass without releasing too much glucose, and then a complete mixture to be added later during SSCF. This would allow for better control of the glucose release rate and concentration level in the process and could potentially enhance xylose conversion.

Continuous operation could also be envisioned, especially at production scale. A continuous viscosity reduction step could, for example, serve as feed source for a following fed-batch SSCF. Continuous fermentation could also be a viable option, although this would likely require a completely separate hydrolysis process, where the remaining solid are removed prior to fermentation, in order to be able to re-circulate yeast.

Chapter 5

Concluding remarks

The aim of this dissertation was to gain a deeper understanding of how to process pretreated biomass at industrially relevant concentrations. The results of the work have contributed to expanding the body of knowledge within this increasingly important field. A comprehensive rheological characterization of steam pretreated spruce has been conducted and connected to the influence of mixing during enzymatic hydrolysis. New insights have been gained by revealing the large differences between materials, most importantly in terms of the power input needed for mixing, as well as the rheological changes during enzymatic hydrolysis. Moreover, since xylose fermentation by recombinant *S. cerevisiae* is affected by the relative ratios of glucose and xylose in the medium, novel process designs could be developed to enhance xylose co-fermentation. The main findings of this dissertation are summarized below.

Part I – Biomass rheology and mixing

- Steam pretreated spruce exhibits non-Newtonian flow behavior, which can be described by a power-law model once flowing. The flow properties and the yield stress are strongly affected by WIS content and particle size distribution, as well as by the processing of the material (i.e. pretreatment method and degree of enzymatic hydrolysis).
- The enzymatic hydrolysis of pretreated spruce is strongly affected by mixing at high WIS content. However, at lower WIS contents, the effect diminishes.
- The effect of agitation on the hydrolysis rate of pretreated spruce remains during scale-up, at least up to a scale of a few cubic meters. Scale down experiments suggest a strong influence of flow regime on the hydrolysis rate.
- The influence of agitation differs greatly between materials, as do the changes in viscosity during hydrolysis. For giant reed, a very quick liquefaction was achieved during hydrolysis, whereas spruce retained its

high viscosity for a much longer time. The difference in liquefaction time most likely explains the absence of mixing effect on the enzymatic hydrolysis of giant reed.

Part II – Process design

- Combining substrate and enzyme feeds enhanced xylose uptake by 25 %, resulting in a 10 % increase in final ethanol titer. This dual feeding helps maintain a low glucose level, while continuously releasing glucose throughout the fermentation, thus promoting xylose uptake.
- In the presence of high levels of acetic acid, xylose consumption can be greatly enhanced by increasing pH. However, the choice of process design determines to what extent this is beneficial.

Thanks to recent progress in research, including the work carried out in this dissertation, the commercialization of 2nd generation bioethanol has now started. Evidently, a long learning curve can be expected and unforeseen processing problems will likely arise. Within this context, the significance of understanding the rheology of pretreated biomass will only grow more important. Despite efforts to understand how mixing influences enzymatic hydrolysis, the mechanistic reason behind its effect is still not fully clear. A possible explanation is the connection to mass transfer limitations, indicated by low Reynolds numbers, although more research is needed to fully outline this.

This work has shown the strong potential of process optimization for enhancing xylose co-consumption. It is hoped to serve as a good starting point, for further process design of efficient combined hydrolysis and fermentations, towards a better production process.

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